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MENDELIAN AND CYTOPLASMIC INHERITANCE IN YEASTS[1]

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The standard ellipsoidal cell of *Saccharomyces cerevisiae* is diploid (Kruis and Šatava, '18; Winge, '35). Under certain conditions its diploid nucleus undergoes meiosis and four haploid spores are produced (Lindegren and Lindegren, '44a; Lindegren and Hamilton, '44). One-, two-, three-, and four-spored asci are found, showing that many accidents may occur during the reduction division (Lindegren and Lindegren, '44a). In our work we select the four-spored asci and dissect out the four spores separately. Each ascospore grown alone produces a small cluster of round haploid cells. Genetical analysis has shown that the ascospores are of two kinds, *a* and *α* (Lindegren and Lindegren, '43b, '43c, '43d; Lindegren, '44). The legitimate diploid vegetative cells are formed by the fusion of *a* and *α* gametes, and these legitimate diploids produce four viable ascospores on reduction, thus completing the cycle. The haplophase cultures, when grown alone, often produce diploid cells by the copulation of two haplophase cells of the same mating type; we call these illegitimate (*a*/*a* and *α*/*α*) diploids because they only rarely produce four viable ascospores. The round-celled haplophase cultures often become stabilized in the haploid condition and gradually become incapable of mating with other forms after being carried in culture for some time (Lindegren and Lindegren, '44b). Asporogenous yeasts such as *Torulopsis* and *Asporomyces* probably originated in this manner.

SEGREGATION AND MUTATION

Haploid yeast cells are much smaller and more variable than diploid cells, varying more both from culture to culture and within a single culture than diploid cells. These differences are also reflected in the colonies, the diploid colonies being larger and more uniform, while haploid cultures produce smaller colonies which are usually rough and generally show considerable variation. The haplophase originates by the reduction of the diplophase at spore formation, and the

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segregation of a heterozygote produces segregants of different genotypes. Yeasts are extraordinarily heterozygous, and a great variation of colonial forms is obtained by the isolation of single ascospore cultures. The haploid segregants are usually rough-colonied; smooth-colonied diploid cells usually produce only rough-colonied haploid segregants. Apparently considerable mutation occurs in the haplophase but generally the original segregant can be distinguished from the secondary mutants when the culture is plated out. At first, the mutants are usually slow-growing and produce small round colonies but on transfer they become adapted and stabilized and their specific colonial character becomes apparent, distinguishing them from the original segregant. Therefore, there are two mechanisms producing variation in yeasts explainable on purely genetical grounds (fig. 1):

(1) *Segregation.*—Segregation of genes of a heterozygous diploid at meiosis produces four spores, each of which develops a different type of colony.

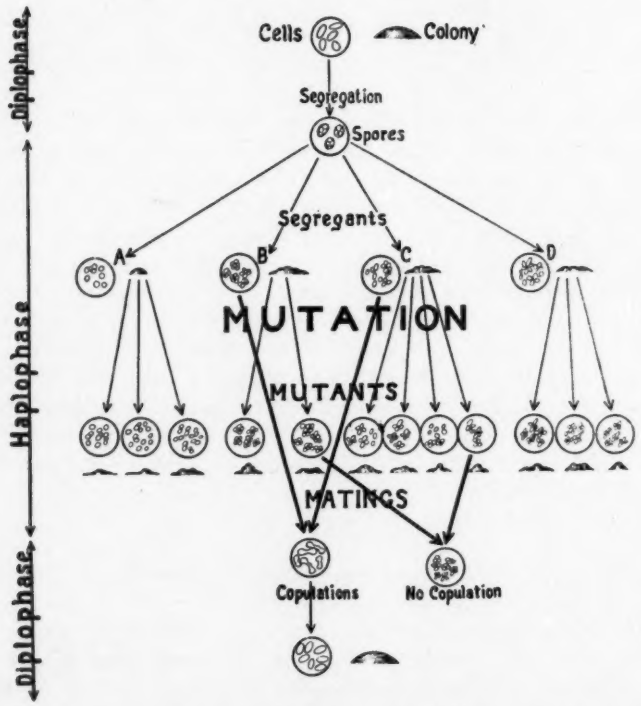

Fig. 1. Diagram showing the effects of segregation and mutation in producing variation in yeasts. The circles show the sizes and shapes of the cells as seen under the microscope and the stippled figures are profiles of the colonies on agar.

(2). *Mutation.*—Mutation in the haplophase enormously increases the variation of colonial forms. It is possible by continued subculturing to develop a tremendous variation of colonial forms from a single haplophase culture, but this usually results in loss of fertility.

DISTINCTION OF HAPLOPHASE AND DIPLOPHASE

Winge ('35) was the first to distinguish clearly between haplophase and diplophase yeast cultures, and we have corroborated his observations with some slight modifications. Workers familiar with other biological material may seriously question the propriety of speaking definitely of haplophase and diplophase in organisms where the cytological facts have not been conclusively demonstrated. I shall therefore summarize all the arguments, Winge's reinforced by ours, for distinguishing haplophase and diplophase. I should preface these rules by saying that over four-fifths of the cultures which one encounters are easily characterized by microscopic examination. They are either obviously haploid or diploid, as shown simply by size, shape, and aggregation of cells. The reasons for classifying them are as follows:

(1) The large vegetative cells which we call "legitimate diploids" produce viable four-spored asci. These spores germinate to produce smaller cells, which we call "haploid." The latter multiply vegetatively, generally maintaining their specific cell-shape and size.

(2) Two of these smaller cells may fuse to produce a large "diploid" cell capable of vegetative multiplication (Winge and Laustsen, '39a, '39b, Lindegren and Lindegren, '43b, '43d). While the large cell is undergoing vegetative reproduction, it retains its characteristic ellipsoidal shape and size. Under certain conditions, this diploid cell can be induced to sporulate. Spores from it in turn produce haploids and the process can be repeated indefinitely.

(3) The large cells which we recognize as diploids are extraordinarily stable in their genetical characteristics when they are grown under conditions in which sporulation does not occur. Transferring the cultures every forty-eight hours in broth is generally sufficient to maintain the vegetative diplophase. Colonies produced by plating out are not sectored; the plates do not show colonial variants. However, when haplophase (single ascospore) cultures of any age are plated out, a variety of colonial variants appear on the plate or the giant colonies are sectored. These facts are consistent with the view that the large cells are diploid, thus minimizing the number of spontaneous mutations found, while in the haplophase every mutant becomes apparent and is easily discovered.

(4) When the diploid cells sporulate to produce haploid cells, there is genetic evidence of a reduction division (Winge and Laustsen, '37). Genetical analysis shows that a single pair of alleles responsible for the two different mating types is segregated at this meiosis. Two a and two α type haplophase cultures are usually obtained from the four single ascospore cultures (Lindegren and Lindegren, '43d). There is also genetic evidence for the segregation of a gene-pair

controlling fermentation of melibiose (Lindegren, Spiegelman, and Lindegren, '44) during the meiosis that precedes spore formation. Also, evidence proving that factors controlling cell shape may be segregated in a hybrid of S. *Bayanus* and S. *cerevisiae* has been accumulated in addition to that previously offered by Winge and Laustsen ('39c) in the balanced heterozygote, *Saccharomycodes Ludwigii*.

(5) Haploid cultures of *a* and *α* mating type have been paired, and the resulting diploid cultures in turn have been induced to sporulate; the haplophases have been tested and found again to fall into the *a* and *α* categories. Matings and tests for this character have been carried through four or five generations in several cases. Similarly, segregation of the gene-pair controlling melibiose fermentation has been observed; the segregants have been tested, mated, and segregation has again been observed in the succeeding generation. Pedigrees of three or four generations are available for many characters.

The above facts seem to prove conclusively that the terms haplophase and diplophase can be used as definitely in speaking of yeasts as of organisms in which the cytological evidence is more complete. The illegitimate diploids provide an exception which is quite familiar to the mycologist. Copulations between haplophases which are usually incapable of copulating on genetical grounds were called "Durchbrechungskopulationen" by Brunswik ('24). Copulations of this type frequently occur in single ascospore cultures and produce diploids which are homozygous for the *a* or *α* factors. With rare exceptions these diploids sporulate poorly, and for this reason we have not studied them intensively. A few single ascospore cultures sporulate well, and some produce large cells that are difficult to classify either as definite haplophases or illegitimate diploids. However, the general rules laid down in the preceding discussion hold very well, and exceptions are not more frequent than one would expect on the basis of mutations, polyploidy, apomixis, or other genetical aberrations.

MATING TYPES

The variability of colonial characters is not paralleled by similar variation of mating-type specificity. With rare exceptions, each haplophase culture belongs to either the *a* or *α* mating type, or is sterile. A considerable number of sterile cultures are found and fertile cultures may become sterile, especially if the haplophase is carried a long time in culture. However, the mating types are differentiated primarily by a single pair of alleles.

A large-scale experiment has indicated that only two principal mating-type alleles are present in S. *cerevisiae*. Figure 2 shows the results of mating 58 different single ascospore cultures derived from a variety of industrial bakers' yeasts. Ly, HD, M, FLD, D, and B represent standard legitimate diploid strains of commercial baking yeasts; 800 is one of the baking strains of yeast obtained by Dr. Wickerham of the Northern Regional Research Laboratory, Peoria, Illinois. Two other cultures are hybrids, one of 800 x L and the other (800 x L) x L. Haplophases isolated from the "(800 x L) x L" hybrid were generally quite fertile.

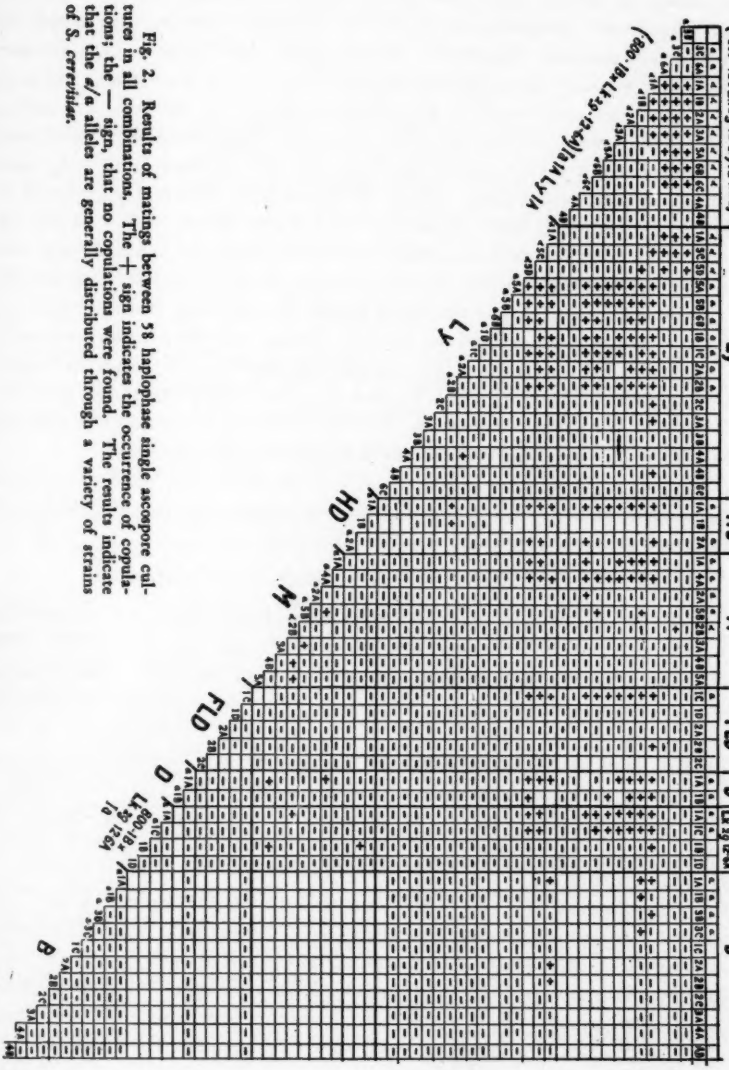

Fig. 2. Results of matings between 58 haplophase single ascospore cultures in all combinations. The + sign indicates the occurrence of copulations; the — sign, that no copulations were found. The results indicate that the *a*/*a* alleles are generally distributed through a variety of strains of *S. cerevisiae*.

Three belong to mating type *a* and seven belong to mating type *α*. Two were sterile. Copulations invariably occurred when an *a* and *α* culture were mated, and haplophase 1A produced illegitimate matings with three other *α* type cultures. The Ly strain was also generally quite fertile. Three *α* type haplophases and seven *a* type haplophases were found in this culture. Only once did an *a* x *α* mating fail to produce fusions. When the "(800 x L) x L" haplophases were mated with the L haplophases, a high degree of fertility was demonstrated, with only seven failures out of forty-eight tests. With this strain also, culture 1A produced illegitimate diploids. This highly fertile culture was also able to mate with 2C, 3A, and 4B, which were incapable of producing fusions with any other culture with which they were tested. When the *a*-haplophases of the L and the "(800 x L) x L" hybrid were outcrossed to the other strains of yeast, only three hybrids were produced in several hundred matings, but outcrossing with the *α* strains was much more successful and resulted in a large number of hybrids. This occurred in spite of the fact that the HD, M, FLD, D, and B cultures were apparently quite infertile among themselves. It appears, therefore, that the *α* strains from this line can be successfully outcrossed to produce hybrids with other strains.

These results demonstrate that the *a*/*α* alleles obey the standard rules of Mendelian inheritance, and that other genes may apparently act as modifiers of mating type, generally resulting in reduced fertility.

CYTOPLASMIC ADAPTATION OF AN ILLEGITIMATE HYBRID

The illegitimate diploids are genetically stable forms because they sporulate rarely and, if transferred frequently in broth, will not sporulate at all. The failure to sporulate eliminates segregation as a cause of variation. Furthermore, diploids are practically free from spontaneous mutations because each locus is "covered" by a dominant normal allele. We have studied adaptation to a specific environment, using an illegitimate diploid. Adaptation to a carbohydrate-peptone mash which contained an unknown substance that inhibited yeasts was studied. The first transfer made from malt medium to this carbohydrate-peptone mash grew very poorly, but adaptation to the new medium always occurred on the second serial transfer.

The malt medium (M) contained 10 per cent malt extract, 0.5 per cent dextrose, 0.5 per cent dried yeast, 1 per cent $CaCO_3$, 3 per cent agar. The carbohydrate-peptone mash medium (C) contained 0.8 per cent sucrose, 0.7 per cent nitrogen-containing solids, 1 per cent $CaCO_3$, 3 per cent agar. M agar is a relatively complete medium which supports an abundant growth of uniformly large colonies. Adaptations of different types of yeast to these media have been reported (Lindegren and Lindegren, '43b). On the first transfer to C agar only a small percentage of cells survives, and the variations in colony-size on this agar are not due to genetic differences. Figure 3 shows the results of plating serially on M and C media.

One of the large colonies from an M plate was suspended in water, and equal

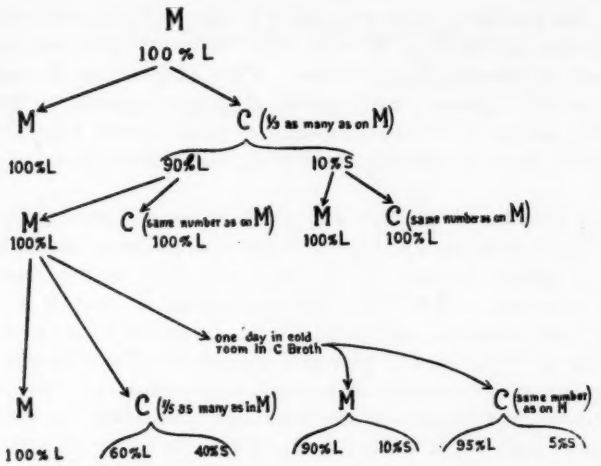

Fig. 3. Diagram showing adaptation of an illegitimate diploid (in which mutations are only rarely expressed) to an unfavorable medium.

amounts of the suspension planted on M and C plates. All the colonies appearing on the M plate were uniformly large. Only about one-third as many colonies appeared on the C plate, and of these 90 per cent were large and 10 per cent were small. In all transfers a large number of cells die; from 10 to 30 per cent of the cells transferred from M to M usually produced colonies, but the mortality is always greater on the first transfer from M to C than on transfers from M to M. Since the genotype is stabilized any selection must be for some difference, independent of the genotype.

A suspension was made of a large colony growing on a C plate, and equal amounts of the suspension plated on M and C plates. The same number of colonies appeared on both plates and all were full size. This proved that the survivors on the C plate were adapted to growth on C agar. A suspension of a small colony gave the same result. The fact that there is no detectable difference between the large and small colonies on C plates indicates that the small colonies are merely slower in development. If a colony is delayed in development, the staling effect of the more rapidly developing colonies on the medium will prevent it from attaining full normal size.

Equal amounts of a suspension from one of the large colonies on an M plate (descended from a colony on a C plate) were plated on M and C plates. Only one-fifth as many colonies appeared on the C plate as on the M plate and both large and small colonies were found. Therefore, cells growing on a C plate (which have become adapted to C agar) lose this adaptation by a single transfer to M agar. This confirms the fact that the first transfer to a C plate did not select genotypes.

It was also possible to adapt the cells to C agar by holding them in C broth in the cold room for two days. A large colony from an M plate was suspended in C broth and held two days in a cold room. When samples from this culture were spread on M and C plates a small number of colonies appeared on both media, although the samples of the untreated culture plated directly from M plates to both M and C plates showed only one-fifth as many colonies on the C as on the M plate.

Since the C medium is obviously unfavorable to the cells coming directly from the M medium it seems probable that the former contains some harmful substance. However, interaction between the cytoplasm and the C medium results in an adaptation apparently enabling the illegitimate diploid to produce a metabolite capable of neutralizing this substance, and this metabolite continues to be produced as long as contact with C medium is maintained. This adaptation must be cytoplasmic because no change has occurred in the genotype. The metabolite which neutralizes the C substance may have been absent from the yeast cells or may be merely increased in amount during adaptation to the C medium. This type of non-genic variation constitutes a complication in the analysis of yeast genetics, and experiments must be designed so that it can be distinguished from the variations resulting from segregation and mutation.

Winge and Laustsen ('40) have demonstrated that a cytoplasmic deficiency may occur in yeasts when a nuclear division is not accompanied simultaneously by a cell division. They have assumed that this condition results from a deficiency of chondriosomes. Their phenomenon is apparently quite different from adaptation of the cell to C medium in which an interaction of substrate and cytoplasm is involved.

MENDELIAN INHERITANCE OF AN ADAPTIVE ENZYME

S. cerevisiae is incapable of fermenting melibiose, and its haploid segregants fail to ferment this sugar even after continued growth in broth containing melibiose. This indicates that mutations enabling the yeasts to ferment the sugar either do not occur in this species or else that they are extremely rare. *S. carlsbergensis* is capable of fermenting melibiose, as are all its haploid segregants. This is the principal character upon which *S. cerevisiae* and *S. carlsbergensis* are differentiated. Figure 4 is a pedigree describing the progenies of matings between these two species (Lindegren, Spiegelman and Lindegren, '44). The data were obtained by growing the cultures in a broth tube containing a smaller inverted tube to collect the gas produced by fermentation. Accumulation of gas in the inverted tube is indicated by a plus sign.

Hybrid I was an interspecific hybrid (*cerevisiae* x *carlsbergensis*) made by mixing melibiose-plus and melibiose-minus haplophase cultures. Three diploid cells isolated after this mating were all capable of fermenting melibiose. Eight asci were dissected from interspecific hybrids, and all the haplophase progeny were tested for the ability to ferment melibiose. The results showed that the haplo-

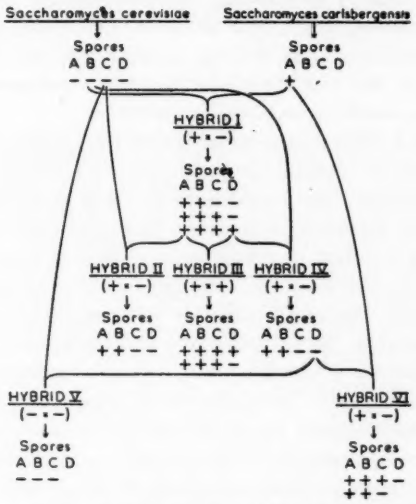

Fig. 4. Fermentation of melibiose by diploid and haploid progeny of *Saccharomyces cerevisiae* x *S. carlsbergensis* hybrids; + sign signifies fermentation of melibiose, — sign, inability to ferment melibiose.

phase cultures from three asci were melibiose +; two asci produced three + and one — culture; and one ascus produced two + and two — cultures. These ratios indicate that more than one gene is involved.

Hybrid II was produced by backcrossing a positive haplophase culture from an ascus producing four + cultures with a negative haplophase culture from *S. cerevisiae*. A regular Mendelian segregation of the progeny shows that the haplophase carried a single gene capable of controlling melibiose fermentation.

Hybrid IV was made by backcrossing a second positive haplophase culture from the same ascus to a negative haplophase culture from *S. cerevisiae*. In this case, a regular Mendelian segregation again shows it also carried a single gene.

Hybrid III was produced by mating the two positive cultures, each of which carried a single gene. Analysis of hybrids II and IV proved that each of these cultures carried a single gene controlling melibiose adaptation. If these genes were alleles, all the haplophase progeny of the hybrid should ferment melibiose. Since two of the twenty haplophase segregants failed to ferment melibiose, the original culture of *S. carlsbergensis* must have contained two different non-allelic loci controlling melibiose fermentation.

Hybrid V was made by backcrossing a negative haplophase segregating from the hybrid to a negative haplophase from *S. cerevisiae*. The three haplophase progeny were all negative.

Hybrid VI was made by backcrossing the same negative culture to a positive haplophase of S. *carlsbergensis*. Five of seven haplophase progeny fermented melibiose, while two failed. This finding, together with the results obtained in hybrid V, confirms the fact that the original haplophase culture of S. *carlsbergensis* possessed two genes controlling melibiose fermentation.

This pedigree is of especial interest because Dr. Spiegelman was able to show that the fermentation of melibiose is under the control of an adaptive enzyme (Kārstrom, '38). Twelve critical cultures were tested in the Warburg apparatus to determine whether fermentation occurred immediately or whether adaptation to the substrate was required, i. e., whether fermentation only occurred after a period of exposure to melibiose. In each case it was found that an adaptive enzyme was involved. The adaptation time was not the same for each strain, which agrees with previous work (Spiegelman and Lindegren, '44), but the time for each is specific and is reproducible under standard conditions.

In the inverted-tube method, part of a clone is seeded into the broth containing melibiose as the carbohydrate source and allowed to grow. Since every mutation in a haplophase population becomes functional immediately, a positive test might not mean that the original clone possessed the fermentative capacity. Selection of a mutant produced during growth in the melibiose solution may have occurred (Spiegelman, Lindegren and Hedgecock, '44). The Warburg tests of the twelve critical cultures excluded this possibility by the fact that in the Warburg apparatus adaptation occurred in a stationary population. If a stationary population exposed to melibiose acquires the ability to ferment the sugar, it can only be due to an interaction between the existing cells and the sugar. Cells which have been adapted to ferment melibiose lose this ability when removed from the substrate and have to be readapted to use it fermentatively.

MAINTENANCE AND INCREASE OF MELIBIOZYMASE IN THE ABSENCE OF THE SPECIFIC GENE

In the preceding experiments on adaptation to melibiose the first contact with the substrate occurred when the culture was transferred to a fermentation tube containing melibiose. A second series of experiments (Spiegelman, Lindegren, and Lindegren, '45) showed that if contact with melibiose were maintained during the growth of the haplophase cultures, during copulation, during growth on the presporulation agar, and during spore formation, all the segregants from heterozygous diploids, such as hybrids II and IV, carrying a single pair of genes, were able to adapt to melibiose fermentation. However, two of the melibiose-plus cultures from each ascus completely lost their ability to ferment melibiose when vigorously dissimilated. This proves that melibiozymase was transferred from the cytoplasm of the heterozygous hybrid which had been maintained on melibiose to the cytoplasm of the haplophase segregants which did not carry the specific gene. Furthermore, the melibiozymase was maintained in the segregants which carried the melibiose-plus gene by an interaction between melibiozymase and melibiose. There-

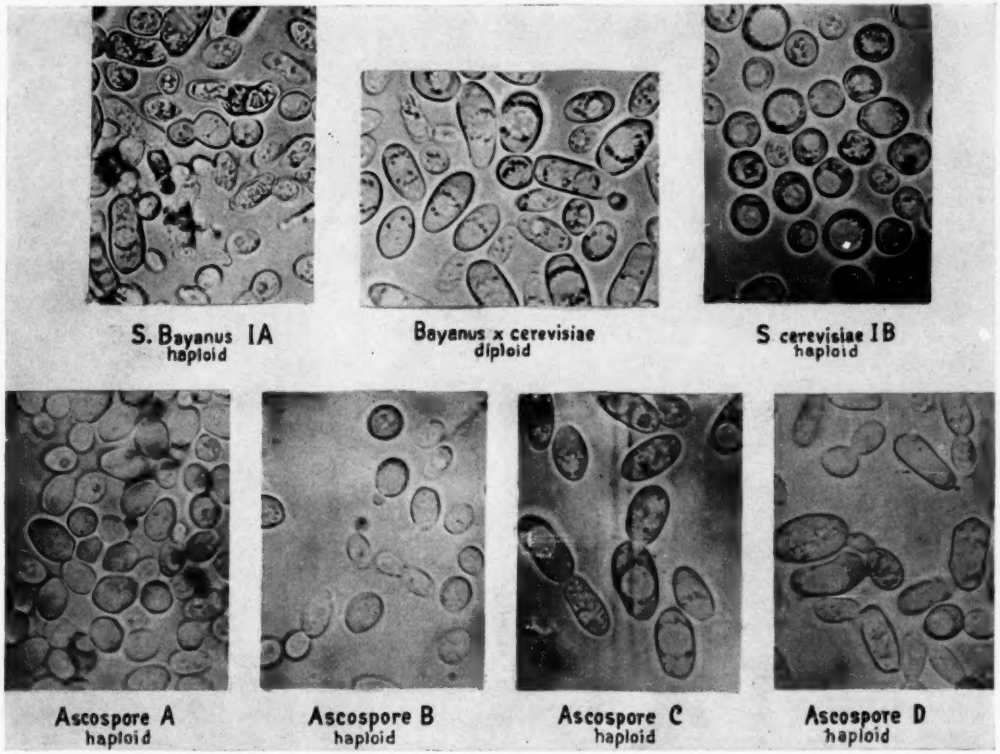

Fig. 5. Photographs of the haplophase cells of *Saccharomyces Bayanus* and S. *cerevisiae* and the diplophase cells of the interspecific hybrid. A, B, C, and D are the haploid cells grown from the four spores of a single ascus of the hybrid.

fore, melibiozymase is a self-perpetuating cytoplasmic entity which is gene-initiated, but the quantitative level of melibiozymase below the maximum depends on an interaction between melibiose and the enzyme and is independent of the gene.

Since S. *carlsbergensis* is homozygous for two pairs of genes which produce melibiozymase, there are four loci in the diplophase of this organism capable of producing this enzyme. There are four corresponding recessive alleles in S. *cerevisiae*, which is probably the most cosmopolitan and best established yeast species. It seems improbable that a successful wild type yeast should carry four functionless genes.

INHERITANCE OF ADAPTATION TO GALACTOSE

The fermentation of galactose by S. *cerevisiae* is due to an adaptive enzyme similar to that produced by S. *carlsbergensis* for the fermentation of melibiose. Since S. *Bayanus* is incapable of fermenting galactose, hybrids between it and S. *cerevisiae* make it possible to study the inheritance of galactose adaptation. There is one advantage in this particular case, namely, that S. *Bayanus* produces large cylindrical cells both in the haplophase and diplophase, providing an additional genetical marker. The hybrid between the large cylindrical (L) gametes of S. *Bayanus* and the round (l) gametes of S. *cerevisiae* produced a large cylindrical (L) diplophase, proving that the *Bayanus*-type cell is dominant. One difficulty is that our culture of S. *Bayanus* sporulated only rarely and only one ascospore of a very large number that was isolated grew. The fact that many of the single ascospore cultures of the hybrid produced viable four-spored asci considerably complicated the genetical analysis. It is notable as an evidence of hybrid vigor that the original hybrid and the progeny all sporulated very abundantly in spite of the poor sporulation of the original S. *Bayanus*.

Figure 6 is a pedigree showing the progenies of a hybrid between S. *Bayanus* and S. *cerevisiae*. All of the haplophase cultures from hybrid IV fermented galactose (+). Half of the single ascospore cultures had large cylindrical (L) cells like S. *Bayanus* and half resembled haplophases of S. *cerevisiae* (l). The large cylindrical *Bayanus*-type cultures fermented galactose more slowly (L + slow) when studied by the inverted-tube technique than did the *cerevisiae*-type (l + fast) cells. The slow fermentation of some of the cultures growing in the fermentation tubes was probably due simply to the slower growth of the *Bayanus*-type segregants.

An analysis of some of the single-ascospore cultures which produced four-spored asci showed that the eight ascospores obtained from two asci isolated from diploid XIII (illegitimate IV-1A) were unable to ferment galactose. This proves that ascospore IV-1A did not carry the gene controlling galactose fermentation but was able to ferment galactose because galactozymase had been carried over cytoplasmically, just as melibiozymase had been carried over in the previous experiments. Our earlier experiments had shown that galactozymase, like melibiozy-

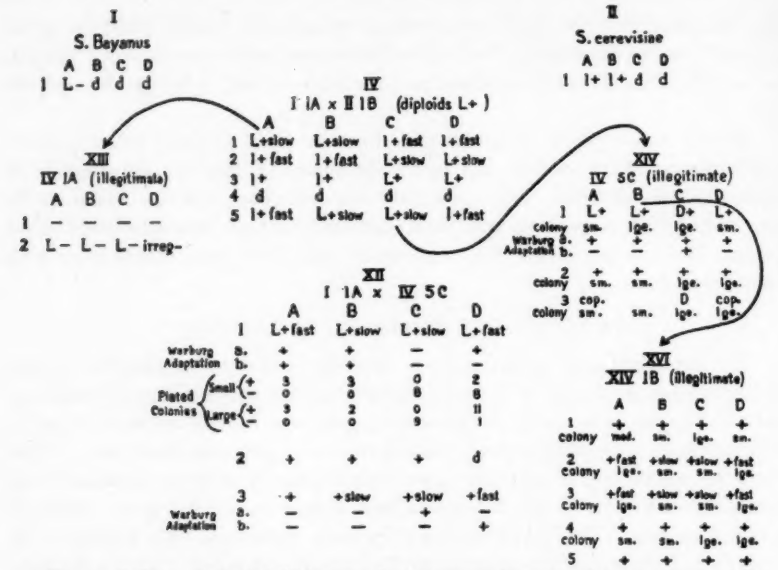

Fig. 6. Pedigree of the hybrid between *Saccharomyces Bayanus* and *S. cerevisiae* and the progeny from it. Discussed in text.

mase, is also substrate-dependent (Spiegelman, Lindegren, and Hedgecock, '44; Spiegelman and Lindegren, '44).

Diploid XIV (IV-5C illegitimate) was L+ and produced viable four-spored asci, and all the single ascospore isolates from it were capable of fermenting galactose. Warburg tests for adaptation to galactose revealed that although all four cultures from ascus 1 adapted within 5 hours on the first trial, three failed to adapt within this period on a second trial. Three asci were dissected from this illegitimate diploid, and each ascus yielded two small- and two large-colony cultures. This suggests that the illegitimate diploid was heterozygous and indicates that copulations in the single ascospore culture had occurred after mutations made the haplophase heterogeneous. Some of the segregants were poorly viable degenerate cells (D) and some produced an abundance of copulations (cop) in the haplophase cultures. These characteristics suggest a similarity to the cytoplasmic deficiencies found in illegitimates by Winge and Laustsen.

Diploid XVI (XIV-1B illegitimate) is the second inbred illegitimate generation derived from IV-5C. Two large- and two small-colony isolates were obtained from each ascus, indicating that the second generation is heterozygous like the first. All the isolates ferment galactose in the inverted-tube tests, and this was confirmed by a second test showing that the original culture, IV-5C, carried the + gene from *S. cerevisiae* together with the *Bayanus*-type cell.

Three asci were dissected from hybrid XII (an L segregant, IV-5C, carrying

the + gene, backcrossed to *S. Bayanus* I-1A, L—). All four ascospores from ascus 1 produced *Bayanus*-celled cultures capable of fermenting galactose. However, when they were grown on sucrose and then tested five hours in the Warburg for adaptation to galactose only A and B proved adaptable in both tests within this period. When all four haploid cultures were plated on agar and single colonies fished and tested for the ability to ferment galactose, A and B produced only fermenting cultures while most of the colonies fished from C and D failed to ferment galactose. These facts indicate that ascus 1 was heterozygous for the +/— alleles.

THE CYTOGENE HYPOTHESIS

These experiments on adaptation show that the ability of different yeasts to adapt themselves to specific substrates is due to a cytoplasmic mechanism. No genetical analysis was available in the case of the adaptation of the illegitimate diploid to C medium, but it was possible to show that genes control the adaptation to melibiose by making hybrids between *S. carlsbergensis* and *S. cerevisiae*, and similarly that genes control the ability to adapt to galactose by making hybrids between *S. cerevisiae* and *S. Bayanus*. In the latter experiments it was clear that although genes initiated the production of the adaptive enzymes, adaptation only occurred by interaction of the cytoplasm of the cells with the specific substrate; and furthermore, the adaptive enzyme, once it had been formed, was self-perpetuating in the presence of the substrate. This was further confirmed in both cases by showing that the adaptive enzyme could be transmitted through the cytoplasm and maintained in cells without the gene.

I propose to call adaptive enzymes of this type *cytogenes*. The fact that a period of exposure to melibiose must occur before the melibiozymase is produced

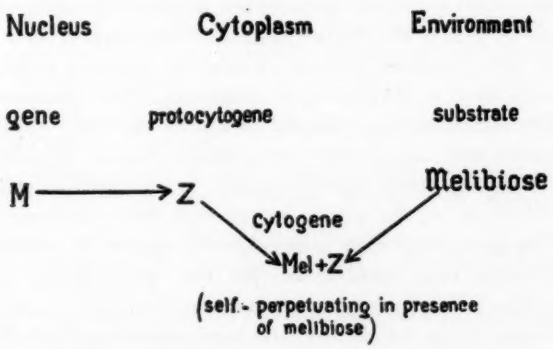

Fig. 7. Diagram explaining the cytogene hypothesis.

suggests that the original product is a relatively non-specific substance which is transformed into melibiozymase when it is "imprinted" by melibiose. The original relatively non-specific substance I propose to call the *protocytogene*. This concept is shown diagrammatically in fig. 7.

If the melibiose-plus gene produces a protocytogene which becomes a specific cytogene, melibiozymase, by being "imprinted" by the melibiose molecule, it is possible that the same locus may be responsible for the production of other cytogenes as well. The original gene-product which becomes specific by contact with the melibiose molecule might presumably become differently specific on contact with some other molecule. Genes are "enzyme factories," but each gene may not necessarily be restricted to the production of a single enzyme. The possibility that a single gene may produce a variety of cytogenes may be a different phenomenon from the one first described by Dobzhansky ('27) as the "manifold effects of a single gene."

DISCUSSION

Darlington ('44) has named certain self-perpetuating cytoplasmic entities which seem to be relatively independent of the genome *plasmagenes*. The cytogene differs fundamentally from the plasmagene, for the former, as defined above, is gene-initiated and substrate-dependent. However, a cytogene might possibly be transformed into a plasmagene by a metabolic mechanism which would synthesize the appropriate molecules within the cell. For example, if *S. cerevisiae* synthesized melibiose, melibiozymase could be maintained permanently in the hybrids as a constitutive enzyme. Such a mechanism might arise by mutation. Therefore, plasmagenes and cytogenes might be phylogenetically related in the following sequence: gene → cytogene → plasmagene.

Darlington has suggested that plasmagenes may evolve into viruses by mutation; however, this implies that plasmagenes are relatively independent entities more or less at the gene level. The preceding discussion suggests that most plasmagenes may be highly dependent on internal substrate for perpetuation rather than relatively independent as Darlington has suggested. The fact that the plasmagenes reproduce exclusively in a specific cytoplasm may mean that their actual existence depends upon contact with some specific type of molecule peculiar to that specific cytoplasm rather than on general "good" growing conditions in the cytoplasm. However, viruses seem to be relatively independent on the substrate and to resemble genes much more than either plasmagenes or cytogenes. Viruses might arise directly from genes rather than from plasmagenes. I ('38) have presented an hypothesis suggesting that viruses may evolve from genes by passage through an insect vector which may have some advantages over the hypothesis suggested by Darlington.

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BIOCHEMICAL GENETICS OF NEUROSPORA

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The production by irradiation of mutant strains of *Neurospora* (Beadle and Tatum, '41) has provided material for the cooperative attack by genetical and biochemical methods on the problem of the mechanism of gene action (Tatum, '44, Horowitz *et al.*, '45, Beadle, '45a). The genetic approach has shown that of the mutant strains so far investigated, both the morphological and biochemical ones are differentiated from normal by single genes. Genetic methods based on crosses and on the formation of heterocaryons have been developed for establishing the allelic or non-allelic nature of specific genes, and for determining the relative dominance of particular biochemical genes (Beadle and Coonradt, '44). The genetic analysis of mutant strains should in time provide data for the location of the mutant genes on chromosome maps. Already quite a few genes have been so located on one or another of the seven chromosomes of *Neurospora* recently demonstrated by McClintock (unpublished).

The biochemical investigations have supported the view that genes controlling biosyntheses of vitamins and amino acids and other biologically important substances in *Neurospora* act through their primary effect in determining the specificity or the production of enzymes involved in carrying on individual steps in the biosyntheses. The mutant strain is perhaps characterized by total or partial failure of enzyme synthesis. Another possibility would be the production of a modified enzyme with altered specificities, which is as a result either inactive or less effective than the normal enzyme in catalyzing the required reaction. If the general concept of a biosynthesis as a sequential series of enzymic reactions is correct, a number of consequences may be predicted which can be tested experimentally. If a given gene affects only one enzyme and therefore only one biochemical reaction, each enzymic step should be controlled by a different gene. Or conversely, two non-allelic genes controlling even the same synthesis must affect different biochemical reactions in that synthesis. It should also follow that an intermediate compound preceding a genetically blocked reaction should be inactive, while one following this point in the biosynthesis might be expected to show the same order of activity as the end product.

The results of investigations have so far supported these predictions. At least seven different genes are known to be involved in the synthesis of arginine. (Srb and Horowitz, '44). Of these, four are concerned in unknown reactions leading to the synthesis of ornithine, two in the conversion of ornithine to citrulline, in which two biochemical steps have been suggested (Krebs, '36), and only one in the conversion of citrulline to arginine, a reaction involving only one obvious step. At least two genes are concerned in the synthesis of tryptophane, one in the production of anthranilic acid and one in the conversion of anthranilic acid

to indole (Tatum, Bonner and Beadle, '44). The latter is then converted to tryptophane by condensation with serine (Tatum and Bonner, '44), a reaction for which no mutant gene has yet been found in *Neurospora*. In the synthesis of thiamin one gene governs the synthesis of thiazole, and another the condensation of thiazole with pyrimidine (Tatum and Bell, unpublished). Another gene is known which apparently controls the synthesis of pantothenic acid from its components, β-alanine and pantoyl-lactone, either one or both of which are inactive for the mutant strain.

A third possible consequence of the original assumptions would be the accumulation of an intermediate the further conversion of which is blocked by the gene mutation. A few examples of this accumulation of intermediate products in *Neurospora* mutant strains are known. The *indoleless* strain 10575 which can form tryptophane from indole but not from anthranilic acid actually produces this latter compound, which must therefore be an intermediate in tryptophane synthesis in *Neurospora* (Tatum, Bonner and Beadle, '44). *Thiazoleless* 18558 produces vitamin pyrimidine, while *thiaminless* 9185 produces both thiamin intermediates but cannot bring about their coupling (Tatum and Bell, unpublished). *Pantothenicless* 5531, which requires intact pantothenic acid, synthesizes both β-alanine and pantoyl-lactone (Tatum, unpublished). Not only has the accumulation of intermediates of known constitution been shown to result from particular gene mutations, but also the production of intermediates of unknown nature. The isolation and identification of these should give some insight into as yet unknown biochemical mechanisms of certain other syntheses. Such an intermediate is produced by *cholineless* 47904 but has choline activity for strain 34486 in which the biosynthesis is blocked at an earlier step (Horowitz, unpublished). Another instance is the production of a nicotinic acid precursor by strain 4540 which is capable of replacing this vitamin for another mutant strain (39401) (Bonner, unpublished). This nicotinic acid precursor instead of accumulating is, under some conditions, further metabolized, apparently with the production of an inactive, intensely colored yellow pigment. An inactive purple-colored compound is apparently formed as the result of a reaction involving an intermediate in the synthesis of adenine (Mitchell, unpublished).

Unfortunately, the accumulation of active intermediates in mutants of *Neurospora* seems to be the exception rather than the rule. In many cases this seems to be due to their lability, which results in the further metabolizing of these products as rapidly as they are formed. Other difficulties in the isolation and identification of these substances are the small amounts produced and the narrow range of cultural conditions under which they can be shown to accumulate. Nevertheless, the results of these investigations of mutant strains suggest that in each a single reaction is blocked, and are consistent with the hypothesis of a one-to-one relation between gene and chemical reaction, through specific enzymes.

A few mutants have been found in which more complex reactions or require-

ments have been indicated. Cases in which the activity of the single essential substance is increased by the addition of a second substance can be interpreted as secondary effects, due to biochemical relations not necessarily connected with the blocked biosynthesis. Examples of this are the sparing action of methionine on *cholineless* (Horowitz and Beadle, '43) and possibly that of thiamin on *pyridoxinless* (Stokes, Foster and Woodward, '43). One well-established actual double requirement resulting from a single gene mutation is that of the two amino acids, isoleucine and valine. The close biochemical relation of these two makes plausible the assumption that their biosyntheses involve either a common precursor or a common enzymatic reaction (Bonner, Tatum and Beadle, '43). This interpretation is consistent with the hypothesis that a one-to-one relation exists between the gene and a given enzyme and primary reaction.

Another instance of a double requirement is known, the basis of which is not so easily interpreted. In strains 17084 and 1090 single-gene mutations apparently block the synthesis of both thiamin intermediates, thiazole and pyrimidine (Tatum and Bell, unpublished). Since there is no obvious biochemical similarity in these compounds the interpretation of the action of the genes concerned on the basis of a single reaction is difficult. One possible interpretation is that the synthesis of only one component is blocked, and that the inactive intermediate which is formed then combines or reacts with the other component or its precursor, thus resulting in an actual deficiency in both. An exogenous supply of both compounds is apparently completely active for these mutant strains. The results of further study of these strains will be of the utmost importance in connection with the general validity of the proposed one-to-one relation of gene and enzyme.

Two fairly common and possibly related phenomena have been met with in *Neurospora* as well as in other micro-organisms. These are cases in which a requirement for a specific substance is altered or dispensed with, the result of prolonged incubation in deficient media, "adaptation" (Bonner, Tatum and Beadle, '43), or as an immediate response to altered cultural conditions. In general, it has been found in *Neurospora* that in both instances the genetic constitution of the modified strains is unaltered. There are two possible explanations for these phenomena. One, suggested for *pyridoxinless* by Stokes *et al.* ('43), is that the gene mutation has resulted in a limitation of the physiological conditions under which the synthesis can be performed by a given mutant strain. This could imply the production by the mutant of an enzyme with more restricted capacities. The other possibility is that there may be alternative mechanisms for carrying on certain syntheses or certain steps in a synthesis, and that these different mechanisms may normally function under different physiological conditions. In this case the gene mutation has resulted in the failure of only one synthetic mechanism. The search for mutants of this type has led to the discovery of a number which require particular substances only under definite conditions, especially at temperatures over 28° C. The substances required by these "temperature" mutants include riboflavin, adenine and uracil (Houlahan and Mitchell, unpublished).

It is possible that the phenomenon of adaptation is essentially analogous to alterations in synthetic capacities in response to external environmental changes. Adaptation could result from either (1) the formation of an adaptive enzyme capable of carrying on an alternative synthetic reaction, the enzyme possibly formed in response to the slow accumulation of the intermediates (substrate), or (2) the lag in adaptation could be due instead to a slow modification in the internal cellular environment, a change eventually leading to the functioning of an alternative reaction, or of the original reaction under the modified conditions in the cells. Further experimental evidence may permit a decision as to whether or not these phenomena involve different reactions. The results should have a direct bearing on the validity of the one-to-one relation of gene and reaction.

The pleiotropic manifestations of certain genes in other organisms may at first glance seem difficult to reconcile with the one-to-one gene to enzyme concept. A possible solution is that relating such effects to multiple functions of the primary gene product (Beadle, '45b). It is possible that certain instances in *Neurospora* may be of value in analyzing some effects of this nature at the biochemical level. In a number of biochemical mutant strains, the primary deficiency is accompanied by a specific sensitivity to other compounds, lacking in other mutant strains and in the wild type. The best analyzed and most striking instance in *Neurospora* is the specific inhibition of *lysineless* by arginine (Doermann, '44). This action of arginine has been interpreted as an inhibition of the utilization of the required lysine by the arginine supplied. The utilization of endogenous lysine in the wild type is not interfered with by exogenous arginine. The different effect of arginine in the two cases may be due to differences in the metabolism of exogenous and endogenous lysine. Other instances also suggest the existence of differences between the physiological action and therefore of the metabolism of exogenous and endogenous materials of biological importance. For *tryptophaneless* mutant strain 10575 indole has a greater molar activity than has tryptophane. This apparently results from the more rapid destruction of added tryptophane than of the tryptophane formed *in situ* from indole. Thiazole has a three- to four-fold greater antipyrithiamin activity than does thiamin (Tatum, unpublished). If pyrithiamin inhibits the utilization of thiamin as suggested by Woolley and White ('43) and by Sarett and Cheldelin ('44), thiamin synthesized in the cell from thiazole must be more effective in antagonizing pyrithiamin than is exogenous thiamin. Houlahan and Mitchell (unpublished) have found with a mutant strain which requires riboflavin at higher temperatures that the growth response to riboflavin is very strongly inhibited by lumichrome, and somewhat by lumiflavin, neither of which inhibits the growth of other strains. These results again suggest differences in the metabolism of endogenous and exogenous substances. The many examples of amino acid inhibitions and antagonisms reported with bacteria and the specific inhibitions noted in *Neurospora* mutant strains, all indicate the complexity of interactions of substances in the living cell, and suggest some of the difficulties to be met with in interpreting data

on growth requirements. These interrelations and interactions may arise in part from differences in the biochemical fates of substances of endogenous and exogenous origin, and may provide a biochemical basis for the explanation of certain types of multiple gene effects.

In conclusion, the results so far obtained with *Neurospora* support the hypothesis that genes concerned in biosyntheses, and probably all genes, act in a primary way by determining the specificity of, or in controlling the production of enzymes. The results also support the view that a one-to-one relation exists between gene and enzyme. At present it seems likely that any apparently multiple gene effects in *Neurospora*, when completely analyzed biochemically and genetically, will be found to be due to common primary reactions, or to secondary interactions not directly related to the action of the mutant gene under consideration.

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GENETIC ASPECTS OF CHANGES IN *STAPHYLOCOCCUS AUREUS* PRODUCING STRAINS RESISTANT TO VARIOUS CONCENTRATIONS OF PENICILLIN

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Fastness or resistance to penicillin and to sulfa drugs is known to occur in strains of bacteria which are sensitive to these chemicals; and in numerous instances on record this new property has been produced *in vitro* under experimental conditions. It is known that the degree of resistance can readily be increased by growing bacteria in a medium containing increasingly higher concentrations of such chemicals, and that the resistant strains so developed retain the property of resistance. My interest in this problem was to obtain quantitative data regarding the origin of resistance and to determine whether or not a genetic interpretation could be given to them. The preliminary summary of these results which has been published (Demerec, '45) indicates that the change to penicillin-resistance in *Staphylococcus aureus* is a genetic change comparable to gene mutation.

Experimental procedure.—The penicillin used in these experiments came from a lot of E. R. Squibb and Sons' preparation of sodium salt of penicillin. This was dissolved in phosphate buffer of pH 6, kept in a refrigerator, and added to the culture medium whenever required by the experiments.

In all experiments a single strain of *Staphylococcus aureus* was used—a culture of which, carrying the number 313, had been obtained from the Northern Regional Research Laboratory in Peoria, Illinois. This same strain has been designated as one of the two international standards for assaying penicillin (Veldee, Herwick and Coghill, '45). In order to reduce the genetic variability of the stock, three cultures were made on agar slants by inoculation from a single colony, and, after 24 hours of incubation, were placed in a refrigerator and used daily as the source of inoculum for all experiments.

Bacteria were grown in a broth medium that did not contain penicillin. They came in contact with the penicillin only when tests for resistance were made. Then certain numbers of bacteria were placed in Petri dishes and mixed with a nutrient agar medium containing the desired concentration of penicillin. These cultures were incubated at 37° C. for 48 hours, and after that period of time bacterial colonies appearing in the medium were counted. The long incubation period was necessary because submerged colonies grow much more slowly in a medium containing penicillin than in one without penicillin.

Results obtained by this method are repeatable; similar numbers of surviving colonies were observed when similar samples of bacteria were taken from the same culture and were plated in a medium containing a certain concentration of penicillin.

Resistance of stock strain.—The strain of *Staphylococcus* used in these experiments was affected by various concentrations of penicillin in the manner shown graphically in fig. 1. The curves given in this figure, representing the results of five experiments, show numbers of survivors when bacteria were grown in penicillin. These six curves are very similar to one another; the heavy line drawn through them represents an average curve.

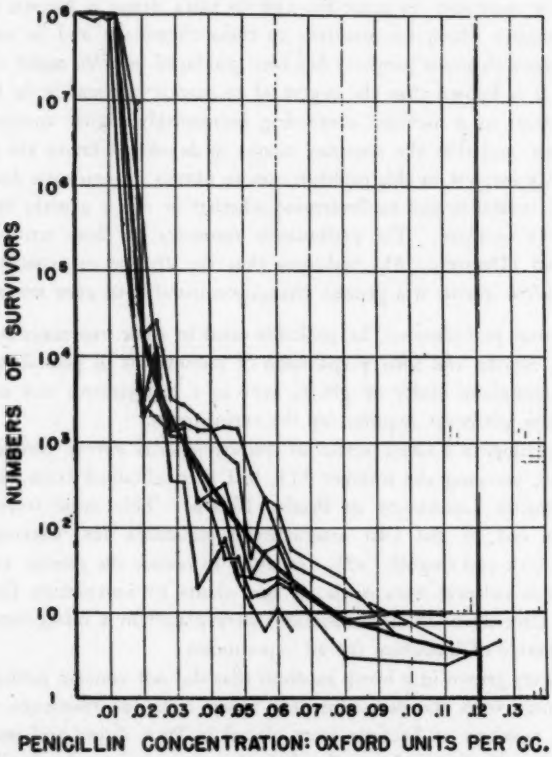

Fig. 1. Numbers of surviving *Staphylococcus aureus* after plating in nutrient agar containing various concentrations of penicillin. The six light curves represent data from six experiments, and the average curve is drawn as a heavy line.

It is evident from fig. 1 that penicillin does not affect these bacteria until a concentration of 0.012 Oxford unit per cc. is reached. That seems to be a threshold concentration for our strain of bacteria. If the concentration is increased by only 0.002 unit over the threshold, the number of surviving bacteria is reduced to 10 per cent. Increase by another 0.002 unit reduces the survivors to 1 per cent;

another similar increase brings the number of survivors down to 1 per 1000; and when a concentration of 0.1 unit per cc. is reached there are on the average only 5 survivors per 100,000,000 bacteria.

The next problem that arose in this study was to find out why some bacteria survive and form colonies in the medium containing penicillin while a great majority of their sister bacteria are eliminated. For this purpose, 32 strains were established—each of them isolated from a single colony that had survived a concentration of 0.064 unit per cc. In order to avoid the possibility of their being members of one clone, each of these colonies was taken from a different experiment. At that concentration (0.064 unit), there are only about 25 survivors per 10^8 bacteria. Tests made with these 32 strains showed that all of them were more resistant to penicillin than was the original stock strain. Survival curves indicated that the threshold for the effectiveness of penicillin had shifted (from the concentration of 0.012 unit per cc. which was the threshold for the original strain) to a region around 0.064 unit per cc. in these selected strains. This result justified the conclusion that survivors in the medium containing 0.064 unit of penicillin per cc. lived because they were resistant to that concentration.

TABLE I

RESISTANCE OF STRAINS ISOLATED FROM COLONIES SURVIVING VARIOUS CONCENTRATIONS OF PENICILLIN

Concentration units/cc.	Number of strains	Resistance higher than parent stock	Resistance similar to parent stock
0.064	32	32	0
0.024	20	18	2
0.022	50	26	24
0.018	54	12	42
0.016	53	10	43

Similar tests were made with 20 strains isolated from cultures containing 0.024 unit of penicillin, with 50 strains isolated from 0.022-unit cultures, 54 strains from 0.018-unit cultures, and 53 strains from 0.016-unit cultures. Results of these tests are summarized in Table I. Of the 20 strains from 0.024-unit cultures, 18 were more resistant than the stock strain, while two had the same degree of resistance as the stock strain. Among the strains isolated at the 0.022 concentration, 52 per cent were resistant; and among those isolated at lower concentrations about 20 per cent were resistant. It is evident that a portion of the survivors on concentrations near the threshold lived not because they were resistant to these concentrations but for some other reason. A possible explanation for the appearance of these survivors is as follows: that occasionally the strength of the

penicillin concentration may be reduced in minute sectors of the medium, owing to some environmental factor, and that in the regions near the threshold concentration such reduction may be sufficient to permit the growth of nonresistant bacteria.

Figure 2 gives survival curves for twelve resistant strains isolated at random at various concentrations of penicillin. The heavy line is the survival curve of the stock strain. Arrows pointing toward it indicate the concentrations at which resistant strains were isolated, and each arrow may be matched up (i. e., solid line, broken line, or dotted line) with the curve of the corresponding resistant strain. It is evident that strains isolated from colonies surviving higher concentrations of penicillin tend to be more resistant than strains isolated from colonies surviving lower concentrations.

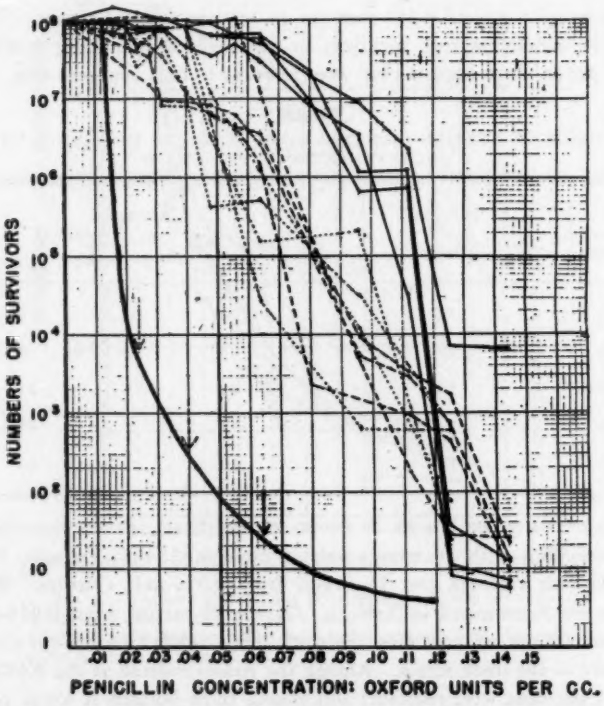

Fig. 2. Heavy line—survival curve for the stock strain; light lines—curves for strains established from colonies surviving the concentrations of penicillin indicated by the arrows.

Inheritance of resistance.—The next problem to be investigated was whether resistance is an inherited characteristic, which persists, or a temporarily acquired feature. Ten strains, isolated from colonies surviving in a concentration of 0.064 unit of penicillin per cc., were passed through 20 transfers in broth; and their survival curves were determined at the beginning of the experiment, at several intervals during the experiment, and at the end of the experiment. The fact that there was no appreciable difference between these curves indicates that the degree of resistance did not change during 20 transfers. Similar results showing the persistance of resistance have been obtained by Schmidt and Sesler ('43) and by Spink, Ferris and Vivino ('44). Thus it is probable that the increase in resistance to penicillin is hereditary, and that it is a stable feature.

Origin of resistance.—The next problem to be investigated dealt with the mechanism of the origin of resistant strains. Here two possibilities were considered: (1) that resistance is an acquired characteristic brought about through some sort of interaction between the bacteria and the penicillin when they are in contact with each other, and (2) that resistance is an inherited characteristic, which originates through mutation and whose origin is independent of penicillin treatment. In the latter case, resistant mutants should occur at random, in a small fraction of a population; and, since a certain concentration of penicillin eliminates all nonresistant individuals, the resistant ones would be selected out from the population by the treatment.

To distinguish between these two possibilities, a modification of the method developed by Luria and Delbrück ('43) in their study of changes in bacteria from bacteriophage-sensitivity to bacteriophage-resistance was used. If the resistance is induced through interaction between the bacteria and penicillin when they are in contact with each other, it would be expected that approximately similar numbers of resistant bacteria would be obtained when samples containing similar numbers of bacteria are plated in nutrient agar containing a certain concentration of penicillin, irrespective of the origin of these samples. The situation would be quite different in the event that the origin of resistance is mutational. In such case, one would expect to obtain similar numbers of resistant colonies only in samples taken from the same culture. If, however, each of the samples comes from a separate culture, and mutations occur at random, then one would expect to obtain a large number of resistant colonies from cultures in which mutation happened to occur early in the growth of the culture and a small number of resistant colonies from cultures in which mutation happened to occur late, assuming that resistant bacteria grow more or less like the normal. If resistance originates by mutation, then, the variation in number of resistant bacteria between samples taken from separate cultures should be much greater than between samples taken from the same culture.

One of the experiments to test these two possibilities was conducted as follows: From the same broth dilution, containing about 300 bacteria per cc., 30 tubes

were prepared with 0.3 cc. of material each, and one tube with about 15 cc. of the material. At the same time, 20 samples of 0.3 cc. each from the same dilution were plated in the medium containing 0.064 unit per cc. of penicillin, to determine if any of the samples contained resistant bacteria. None was observed; and therefore it was reasonable to assume that each culture was started with an inoculum consisting of susceptible bacteria only. Cultures were incubated at 37° C. for about 18 hours, and during that time the number of bacteria increased to about 2×10^8 per cc.; that is, in the 30 small cultures, from about 100 to about 6.6×10^7. The entire contents of each of the 30 tubes were plated in a Petri dish with 0.064 unit of penicillin per cc. of the culture medium; and 20 samples of 0.3 cc. each were taken from the large tube and were similarly plated with the medium containing 0.064 unit of penicillin per cc. In each of these 50 platings about 6.6×10^7 bacteria were placed in medium containing an identical concentration of penicillin; therefore, if resistance develops through interaction between bacteria and penicillin, one would expect to find on each plate a similar number of resistant colonies. However, if resistance originates through mutation, then one would expect that the 20 samples taken from the same culture would give similar numbers of resistant colonies, while an appreciable degree of variation in number of resistant colonies would be expected among samples taken from the different cultures. The results (Demerec, '45) show very slight variation in number of resistant colonies among the 20 samples taken from one culture. The extreme variants are 16 and 38; the average number of colonies per culture is 28.9; the variance is 39.8, χ^2 is 22.7, and P is 0.3. On the other hand, the number of resistant colonies per sample taken from independent cultures varies greatly. The extreme variants are 9 and 839, the average is 120, the variance is 42,718, χ^2 is 10,670, and the probability that such a distribution may be due to sampling is extremely small.

The results of this experiment, therefore, favor the assumption that resistance to certain concentrations of penicillin originates through mutation, and that resistant bacteria may be found in any large population. In this case, the proportion of resistant bacteria depends on the mutation rate.

Effect of selection on degree of resistance.—The strain of *Staphylococcus aureus*, NRRL-313, is eliminated if grown in a medium containing more than 0.15 Oxford unit of penicillin per cc. As has been mentioned earlier, an average of 25 out of 10^8 bacteria survived the concentration of 0.064 unit per cc. From these, strains more resistant than the original strain were established. In strains developed from survivors on an 0.125 concentration, there were individuals resistant to 0.25 unit; strains from these latter survivors had individuals resistant to 0.5 unit; strains from these included individuals resistant to 4 units; and from these a strain was isolated that was not affected by a concentration of 250 units of penicillin per cc. of the agar medium.

The result of this type of selection on increase of resistance to penicillin is

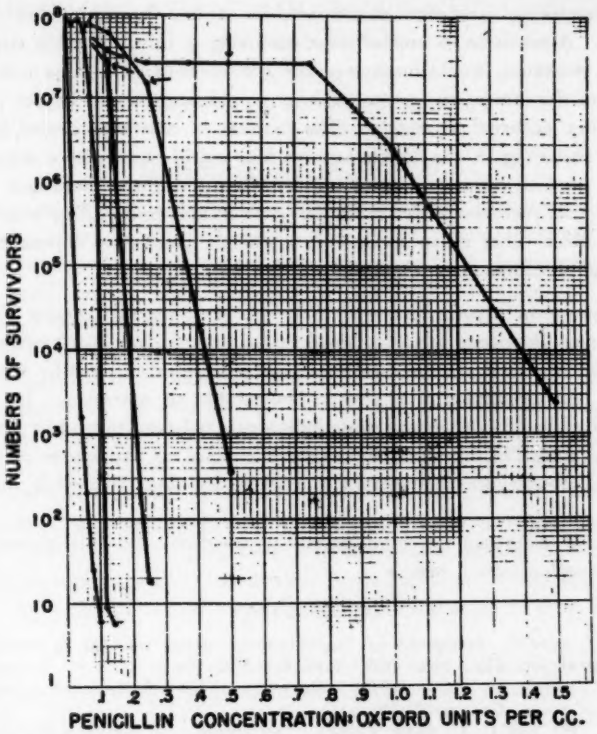

Fig. 3. Numbers of survivors in various concentrations of penicillin, for the stock strain and for four resistant strains developed through repeated selection.

graphically shown in fig. 3. The first curve in this figure represents the number of survivors in various concentrations of penicillin for the stock line of *Staphylococcus;* the second curve represents survivors in a strain isolated from a concentration of 0.064 unit; the third curve shows survivors of a strain isolated from an 0.125 concentration; the fourth is for a strain isolated at an 0.25 concentration; and the fifth for a strain isolated at 0.5 unit. The arrows indicate, on each curve, the concentration at which the strain was isolated. It is evident that the building up of resistance is more rapid with each selection step; the increase appears to be exponential.

Discussion.—The evidence presented here indicates that resistance is a complex characteristic, and that it must involve a number of mutations. If it is assumed that genes are responsible for these mutations, a number of genic changes must be involved. This assumption can readily explain the increase of resistance obtained through selection. A mutation in one of the several

genes determining resistance would produce a line having a low degree of resistance. A mutation in another gene, occurring in this line which already had a degree of resistance, would produce a line (double mutant) whose resistance was higher than the sum of the resistances that would be produced by the two mutations if they occurred separately. The increase in resistance caused by another mutation occurring in the double-mutant line would raise it to a degree greater than the sum of resistances produced by the same three mutations occurring separately; and every successive mutation in a multiple-mutant line would produce a similar effect—that is, an increase in resistance proceeding exponentially with the number of mutant genes involved.

Summary.—In experiments with *Staphylococcus aureus*, strains resistant to penicillin were developed which retained that property after 20 transfers in broth.

Experimental evidence indicates that resistance is not induced by the action of penicillin, but originates as a change comparable to mutation. In any large population of bacteria there are some individuals resistant to certain low concentrations of penicillin. If this population is exposed to the action of such concentrations of penicillin, nonresistant individuals are eliminated while the resistant survive.

Degree of resistance can be increased by selection; and this increase is more rapid with each selection step.

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THE PHYSIOLOGY AND GENETIC SIGNIFICANCE OF ENZYMATIC ADAPTATION[1]

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THE PHENOMENON OF ADAPTATION

Wortmann (1882) showed that certain bacterial species could produce amylase only when grown in the presence of starch. Since these early observations many more of a similar nature have been made. The bacteriological literature in particular contains innumerable instances of so-called "training" phenomena of the most varied kinds. Recent reviews by Karström ('38), Yudkin ('38), Rahn ('38), Stephenson ('39), Linderstrom-Lang ('40), Dubos ('40), and Gale ('43) summarize the available data.

The essentials of the phenomenon may be stated in the following terms: a population of cells placed in contact with some substrate acquires, after the lapse of some time, the enzymes necessary to metabolize the added substrate. The removal of the substrate leads to the disappearance of the enzyme system it evoked.

Karström ('38) designated as "adaptive" those enzymes which are produced as a specific response to the presence of the homologous substrate. Such enzymes were differentiated from the "constitutive" ones which are always formed by the cells of a given species, regardless of the presence or absence of their homologous substrates.

Because of its convenience, Karström's terminology has been widely adopted. In recent years, however, it has become increasingly clear that it is not adequate for the description of the facts. In the first place, the classification raises the obvious difficulty that the ease with which a given enzyme is detected in low amounts will determine the category in which it is placed. In addition, enzymes which have been labeled constitutive undergo wide fluctuations in the presence and absence of their substrate. Thus, the invertase content of *B. coli* rises to 452 (Stephenson, '39) in the presence of sucrose and falls to values lying 12.4 and 39 in its absence. Again the $Q_{glucose}$ value of *B. coli* is about 1,000 for organisms grown in the presence of glucose and about 190 for those grown in lactate medium (Stephenson and Gale, '37). In all carefully examined instances where enzyme-substrate relations are known, substrate has stimulated or stabilized its enzyme. The only claim for independence of enzyme level from its substrate was made by Quastel ('37), who reported that glucose stimulated urease formation whereas urea suppressed it. However, Epps and Gale ('42) reinvestigated the problem and showed that the differences Quastel observed depended on the fact that ex-

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traction was done in the absence of substrate. They found that growth in the presence of urea stabilized the urease content of cells.

While exceptions may be found by future study, there does exist a relatively large group of well-defined enzyme systems which respond positively to their specific substrates. In the case of the "adaptive" enzymes, the response is marked and the enzymes fall to zero or near zero levels in the absence of substrate. These enzymes seem to differ from the constitutive ones solely in their relatively greater instability in the absence of substrate. It seems questionable, from this point of view, whether classification into "adaptive" and "constitutive", implying as these terms do a difference in origin and function, is fruitful or even valid. This same point of view implies that enzymatic "adaptations" are but quantitatively exaggerated instances of a more general phenomenon resulting from the effects of substrates on the synthesis and stability of their enzymes.

While we shall in the present discussion use the term "adaptive" in connection with enzyme formation, it should be emphasized that this is not meant to imply the *de novo* induction by substrate of the enzyme concerned. The term is used here to describe the situation in which an enzyme responds by increasing in the presence of its substrate and decreasing in its absence.

From the standpoint of genetics, enzymatic adaptation has several interesting possibilities. Thus far, attacks on the problem of the nature of gene action has had to depend, for the most part, on the study of the final end products of enzymatic activity. It has generally been assumed that genes determine phenotype by virtue of their control of enzymatic constitution. If this be true, the process of adaptation presents an unique opportunity for examining certain details of gene action. In particular, it is reasonable to hope that such studies could delineate the nature of the control exercised by genes over enzyme activity. It is the purpose of this paper to present some data bearing on this problem.

We shall confine our attention to galactozymase and melibiozymase activities and their variations in yeast cells.

BIOLOGICAL MECHANISMS OF POPULATIONAL ADAPTATIONS

Large numbers of individuals are always involved in adaptation experiments, and it is inevitable that attempts to elucidate further the biological nature of these modifications encounter a basic problem common to all studies of physiological changes in large populations. A comparative biochemical study of large populations always involves over-all populational characteristics. This necessarily introduces difficulties into the interpretations of any observed changes in physiological properties. The mechanisms available to an individual cell for adapting itself to an environmental change are limited by its genome and the physiological flexibility permitted by its particular degree of specialization. When, however, the adaptation of a population of cells is being considered, there must be added to the physiological pliability of its members the genetic plasticity of the group in terms of the numbers and kinds of variants it is capable of producing.

Because of this composite nature of populational adaptability, it is clear that in any given case the same end result can be obtained by any one of the following mechanisms: (1) The natural selection of existent variants with the desired characteristics from a genotypically heterogeneous population; (2) induction of a new (as far as measurements are concerned) enzyme by the substrate in all the members of a homogeneous population; and (3) a combination of natural selection and the action of mechanism (2) on those selected.

It was difficult to resolve these questions with bacteria since genetic control over their populations is not attainable. In two instances, however, a decision on the biological mechanism involved was possible. Lewis ('34) showed that the ability of so-called "trained strains" of *B. coli* to ferment lactose originated through the natural selection of a spontaneous variant which was always present in the original culture in the ratio of about $1:1 \times 10^5$. Stephenson and Stickland, ('33) were able to demonstrate the formation of hydrogenlyase in the presence of formate in non-dividing cultures of *B. coli.*

It is clear that a considerable advantage would be gained if it were possible to study this phenomenon with microorganismic populations whose genetics could be controlled. Aside from the obvious possibility of examining the genetics of the process, the study of its physiology could be enormously simplified. Reproducibility of the measurements would thus be assured and the complications of natural selection, which are always present when dealing with genetically heterogeneous material, could be avoided. The opportunity of using genetically controllable material was provided by the fundamental work of Winge and Laustsen ('37, '38, '39a), in Denmark, and the Lindegrens ('43a, b, c), in this country, on the genetics and life cycle of the yeasts.

THE GENETIC CONTROL OF GALACTOZYMASE FORMATION IN YEAST POPULATIONS

Dienert ('00) was one of the first to describe a well-defined example of populational adaptation in the yeasts. He showed that suspensions of yeast cells could acquire the enzymatic apparatus necessary to ferment galactose when placed in contact with it. Since its discovery by Dienert this particular problem has been investigated by numerous workers. Armstrong ('05) confirmed Dienert's findings and further found that some yeasts were incapable of acquiring this physiological property, no matter how long they were cultured in the presence of galactose. Slator ('08) showed that those yeasts capable of fermenting galactose possess this ability only after they had been acclimatized by culture in its presence. No yeast he investigated was able to ferment this hexose immediatley upon being introduced to a medium containing it. There was always an induction period of variable length connected with the acquisition of this property.

Several attempts were made to decide whether natural selection or a direct interaction between the galactose and the cytoplasm was involved in the appearance of galactozymase. Sohngen and Coolhaas ('24) grew their yeast cultures at

30° C. and measured enzymatic activity at 38° C. to avoid cell division during the measurement of CO_2 evolution. They concluded, from their experiments, that the production of galactozymase parallels the formation of new cells. In addition, they confirmed Kluyver's ('14) findings that at 38° C., at which temperature cell division is completely inhibited, no adaptation takes place. Other investigators also tried to obtain adaptation in the absence of cell division, since this would clearly exclude the operation of natural selection as a causal agent in effecting the change. Euler and Nilsson ('25) and later Euler and Jansson ('27), in a more thorough investigation, tried, without success, to adapt yeast in the presence of 0.5 per cent phenol to inhibit cell division. The failure of the above-mentioned authors to find adaptation in the complete absence of cell division cannot be taken as conclusive evidence that no such phenomenon could exist. Especially is this true in those cases where suppression was obtained by such agents as heat or cellular poisons. It is not unlikely that in cultures where this "ideal" had been reached, the physiological state of the cells was such that their ability to synthesize new enzymes had been lost along with their ability to divide.

Stephenson and Yudkin ('36) concluded from their experiments that the production of galactozymase in yeast cultures need not involve the formation of new cells. This conclusion was based on the observation that the ability to evolve CO_2 anaerobically, from a medium containing galactose, was acquired in a period when the total and viable count remained constant. These findings were apparently in direct contradiction with those of previous workers and in particular of Sohngen and Coolhaas ('24).

Before undertaking any detailed study of the physiology of this adaptation, it was clearly necessary to resolve this difficulty. It is evident from the nature of the problem of populational adaptability that one of the crucial problems at issue is the phenotypic homogeneity or heterogeneity of the starting population. The possibility of attacking the problem from this point of view was provided by the use of known haploid and diploid strains, thus permitting genetic control over the populations being studied.

Two strains of *Saccharomyces cerevisiae*, Db23B and LK2G12, both of which could acquire the ability to ferment galactose when grown in its presence, were selected for study. Strain Db23B, which was known to be a haploid and therefore genetically unstable, was shown (Spiegelman, Lindegren and Hedgecock, '44) to be phenotypically heterogeneous with respect to galactose fermentation. Some individuals in populations derived from this strain could not adapt to ferment galactose, whereas others could. Strain LK2G12, on the other hand, which was known to be diploid, was uniformly homogeneous in that all of its individuals were able to acquire the capacity for the fermentative utilization of galactose on standing in contact with the sugar. The adaptive behavior of these two strains followed what would be expected from the data obtained on their phenotypic

characteristics. Populations of Db23B, starting with a low percentage of the fermenting type, could increase their enzymatic activity only through the mechanism of cell division and the subsequent selection in favor of the galactose fermenters. It was shown (Spiegelman and Lindegren, '44) that the kinetics of adaptation by Db23B populations were in agreement with the natural selection hypothesis. Using the appropriate strains, experiments were performed which sought to duplicate the findings reported by Sohngen and Coolhaas and those reported by Stephenson and Yudkin. The results are summarized in fig. 1.

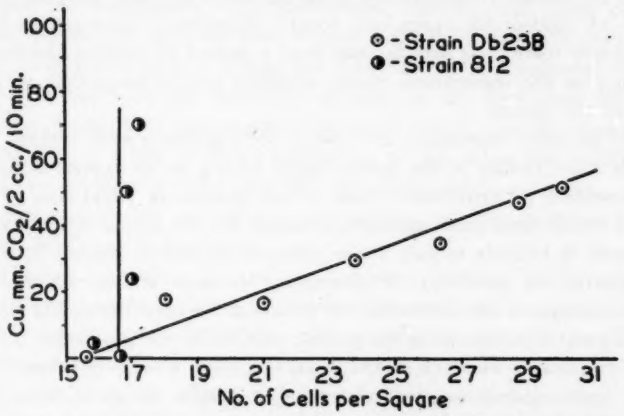


Fig. 1. A comparison of the variation of enzyme activity (expressed as rate of anaerobic CO_2 production) and the number of cells in adapting cultures of haploid (Db23B) and diploid (812) strains.

It is strikingly apparent that populations of the haploid strain, Db23B, increase their enzyme activity by virtue of the new cells arising during the experimental period, and agree with Sohngen and Coolhaas. On the other hand, the measured activity of the diploid 812 population was, in the period examined, virtually independent of cell number. This strain was able to increase its activity from zero to an activity level of 70, while maintaining its population at the same density. The results with this strain thus confirm Stephenson and Yudkin.

From these results it is clear that the contradiction noted is only an apparent one and is probably due to the differences in the genetic background and phenotypic constitution of the strains employed. The conclusion may also be drawn that it is futile to attempt to decide, as some previous authors have tried to do, between the "natural selection" hypothesis and the one of "direct cytoplasmic interaction", as *the* explanation for the production of some one adaptive enzyme. The particular biological mechanism involved in the production of a given enzyme or enzyme system in a population of cells is a characteristic of the strain being examined, rather than of the enzyme system itself. Such questions cannot be answered without referring to the genetic background and stability of the popula-

tion being studied. It is also evident that such strains as LK2G12 and 812 possess three important characteristics of immense value for investigations into the physiology of enzyme synthesis, namely, (1) a genome which permits the synthesis of the enzyme being studied; (2) the genetic stability to insure reproducibility of the physiological characteristics of the populations, and (3) the ability to adapt without cell division.

THE GENETIC BASIS OF INABILITY TO ADAPT TO GALACTOSE FERMENTATION

From the earliest investigations by Armstrong ('05) and Kluyver ('14), the existence of unadaptable yeasts was noted. Subsequent investigations have uncovered many more. As may be seen from a perusal of Stelling-Dekker's ('31) monograph on the sporogenous yeasts, examples of non-fermenters of galactose exist in all the genera.

It did not seem improbable, in view of our experience with Db23B, that the failure to adapt certain of the strains might be due to incomplete utilization of their mutational potentialities. Thus, a non-sporulating population of diploids or one in which some other mechanism existed for the suppression of the haplophase would be unlikely to gain a new character or lose an old one by mutation. This suggested the possibility of attempting to adapt strains, which had been previously labeled as non-fermenters of galactose, by encouraging the production of haploids and thus disturbing the genetic stability of the population. From the point of view of the life cycle of the yeast, this could usually be accomplished by inducing heavy sporulation and allowing germination to occur, thus releasing haploid cells into the population.

Such experiments were performed (Spiegelman and Lindegren, '45) with three yeast types, *Schizosaccharomyces Pombe*, *Schizosaccharomyces octosporus*, and *Saccharomycodes Ludwigii*. All three were investigated by Armstrong ('05), who concluded that they were incapable of adaptation to galactose fermentation. In addition to the fact that they have been studied more thoroughly than other non-fermenters of galactose, they were selected for another advantage, which is of some importance from a comparative point of view. *Sch. Pombe* can, without any difficulty, exist in the haplophase. This is not true of *Sch. octosporus* and still less so for *Saccharomycodes Ludwigii*.

The mechanisms of suppression of the haplophase in the latter two strains differ. While *Sch. octosporus* sporulates with ease, the haploids which result from the germination fuse rapidly to produce diploid cells. When spores from four- and eight-spored asci from the stock culture were planted, they all grew and every single-spore culture thus obtained sporulated on the agar in less than 48 hours. The further spore analysis of this strain indicates that the diploid stock culture was completely homozygous and that, unlike *S. cerevisiae*, the production of viable spores apparently does not depend on the prëexistence of a heterozygous nucleus. The sporulation of single-spore cultures was never observed in the *S. cerevisiae* strains used in the adaptation studies. The fact that it does occur in

Sch. octosporus is an indication of heavy diploidization which is confirmed by direct microscopic observation. Isolated non-fusing haploid cells are rarely seen in suspensions of single-spore cultures of *Sch. octosporus*. This process of immediate fusion effectively suppresses the haplophase, and in these cultures genetic variations come mainly from recombinations. This source of variation would obviously not be effective in populations which are homozygous for the recessive.

The suppression mechanism is even more highly developed in *Saccharomycodes Ludwigii*. Guilliermond ('03) reported that this yeast usually forms four spores without previous conjugation. On germination, however, the spores conjugate within the mother cell, two by two, so that only two vegetative cells emerge from each four-spored ascus. Winge and Laustsen ('39b) confirmed these observations and described the successful isolation of the haplophase by micromanipulative dissection of the ascospore and separation of the four spores before germination. As a result of their examination of the haplophase cultures they concluded that *Saccharomycodes Ludwigii* was a balanced heterozygote. The net result of the germination mechanism is the almost complete suppression of the haplophase under normal conditions.

When heavily sporulating (20 per cent and above) cultures of *Schizosaccharomyces Pombe* were seeded into 8 per cent galactose, 2 per cent glucose media, adaptable populations were recovered. The results are summarized in Table I. Exactly similar experiments with *Schizosaccharomyces octosporus* and *Saccharomycodes Ludwigii* failed. These failures are understandable in terms of the inability of the latter strains to express the mutational potentialities of their haplophases. It might also be noted that adaptable cultures were never recovered from *Sch. Pombe* in the absence of heavy sporulation no matter how long contact with galactose was maintained.

It is clear from these experiments that inability to adapt is in some cases due to the genetic stability conferred by diploidy. It may, however, be doubted whether all that is required for populational adaptation is the breakdown of the genetic stability of the unadaptable strain. It is conceivable that the haplophase of a particular strain might not contain within its mutational potentialities the ability to mutate in the direction of, for example, galactose fermentation. That such indeed could be the case was shown by the isolation of three haploid strains (Spiegelman and Lindegren, '45) which could not mutate towards galactose fermentation although kept in contact with the sugar over a four-month period. During this same period they were, however, throwing off numerous physiological and morphological mutants of various kinds.

As was noted by Lindegren ('45), preliminary experiments on hybrids between *S. Bayanus* and *S. cerevisiae* clearly indicate a typical genetic control of adaptability and non-adaptability to galactose fermentation.

TABLE I

ADAPTATION OF *SCHIZOSACCHAROMYCES POMBE* TO GALACTOSE FERMENTATION

Experiment	Origin of heavily sporulating cultures (20% and above)	Days required for appearance of adaptation
1	20-day broth culture	8
2	20-day broth culture	6
3	24-day agar slant	12
4	Gypsum Block	2
5	Gypsum Block	6
6	Gypsum Block	4
7	Gypsum Block	4

BIOCHEMICAL ASPECTS OF ADAPTATION

Before undertaking an analysis of the genetic implications of the phenomenon, there are certain questions it would be desirable to answer as completely as the data allow. These questions would involve, among other things, the existent evidence for enzyme formation, possible biochemical functions of the induced enzymes, and connection of the adaptation with the over-all metabolic activity of the cell.

Implicit in the discussion presented here, as well as in the entire literature of the so-called "adaptive" enzymes, is the assumption that when a cell is placed in contact with some substrate and acquires, during the course of time, the ability to metabolize the added substrate, a new enzyme must have made its appearance. This assumption stems, of course, from the innumerable observations that every metabolic process requires an enzyme. Direct proof that an enzyme is synthesized would involve its isolation from the adapted cells and is not available in any known case of adaptation. Such proof, in any case, would have to be preceded by a determination of the number and functions of the enzymes induced by the substrate. At present the best that can be offered is evidence of enzyme activity in cell extracts after adaptation. In the case of both galactozymase and melibiozymase, sufficient work has been done to remove any reasonable doubt on the question of enzyme involvement in the adaptation. A delayed penetration into the cell can certainly not be invoked as the explanation of the induction period in galactose fermentation. It was shown (Spiegelman, '45a) that galactose actually enters the cell immediately and is metabolized by a purely aerobic mechanism in the preadaptive period before the fermentative enzymes make their appearance. Further, it has been found earlier (Harden and Norris, '10) that yeast juice and maceration extract prepared from adapted yeasts grown on galactose were able to

ferment galactose. Similar preparations from glucose-grown cultures were inactive. These experiments were repeated and confirmed with our own strains using toluol cytolysates. It is clear from these experiments that something, possessing galactose-fermenting capacity, can be extracted from cells after adaptation which was not there before. Experiments of the same type on cell extracts were performed in adaptations to melibiose fermentation. Here again activity could be demonstrated in the cytolysate only after adaptation was established in the intact cells. It may be noted here that all such extracts were made in the presence of substrate.

Certainly a question of prime importance is the nature of the enzymatic changes necessary for the newly acquired metabolic property. Is a whole new set of enzymes required? Or, is only one or two formed which would transform the sugar into one utilizable by the glucozymase system?

In the case of melibiozymase, it seems most probable that a single enzyme only is formed which splits melibiose into glucose and galactose. An enzyme of this kind has been demonstrated in emulsin preparations by Kuhn ('23), who called it melibiase. Little direct work on this enzyme has as yet been done.

Because of the relative importance of galactose in mammalian physiology, a considerable amount of work has been done on the nature of the enzymatic change which permits a yeast cell to ferment this hexose. Harden and Norris ('10) found that a fermenting mixture of yeast-juice (from an adapted yeast) and galactose reacted with added phosphate in a manner similar to ordinary yeast juice and glucose, although a longer induction period was necessary. The rate of CO_2 formation was accelerated, an extra amount of CO_2 equivalent to the phosphate added was evolved, and the rate then again became normal. The phosphate was converted into an organic form not precipitable by magnesium citrate mixture. Euler and Jansson ('27) showed that when dried adapted yeasts are washed, they fail to ferment galactose. However, such preparations can be reactivated to galactose by adding the co-enzyme prepared from unadapted yeast. They were thus able to exclude the possibility that adaptation is concerned with the modification of the existent cozymase or the formation of a new one. Nilsson ('43) was able to isolate from the products of the fermentation of galactose by a sample of dried adapted yeast, a diphosphoric ester which, in its elementary analysis and specific rotation, closely resembled the hexosediphosphate formed during the fermentation of glucose, fructose and mannose.

In order to explain the formation of the same diphosphate from galactose as from the normally fermentable sugars, Nilsson ('43) suggests that the fermentation of a hexose involved the splitting of the monophosphate ester into a triose and a triose phosphate ester. This would destroy the spatial specificity of the fourth carbon atom in the galactose molecule and the resulting phosphorylated triose could be built up into fructose diphosphate. If this were true, it would be necessary to postulate the formation of only one enzyme in the adaptation to galactose fermentation.

It is true that Meyerhof and Lohmann ('34) have shown the existence of an enzyme (zymohexase) in yeast and muscle preparations capable of converting triosemonophosphoric ester into fructose diphosphate. Nevertheless, experimental support for Nilsson's scheme of fermentation is relatively weak. He assumes that the formation of fructose-di-phosphate is an artifact and that the fermentation proceeds from his postulated split of the hexose monophosphate. He bases this on the observation that fructose diphosphate is fermented much more slowly than fructose. However, this can be explained in terms of the classical Meyerhof-Parnas fermentative scheme on the basis of the unavailability of phosphate acceptors. Thus, Meyerhof and Lohmann ('27) found a CO_2 production of only 25 per cent of the calculated values when maceration extract fermented Robison esters. Warburg and Christian ('39) have shown that the partial dephosphyorylation of 1:3-diphosphoglyceric acid to 3-phosphyglyceric acid by the hexokinase-adenosine diphosphate-hexose system may become limiting in the fermentative process.

Cattaneo ('33) has obtained additional evidence pointing to the convergence of the fermentation paths of galactose and glucose by isolating phosphoglyceric acid from the phosphorylated products formed during the fermentation of galactose by preparations of adapted yeast in the presence of added phosphate, acetaldehyde and sodium fluoride. Grant ('35) reinvestigated this problem and confirmed the work of previous investigators on the role of phosphorylations in the metabolism of galactose by adapted yeast. He was able to establish, with some certainty, that the phosphorylated products which accumulated during the fermentation of galactose are not the esters of this sugar but of glucose and fructose. The hexosediphosphoric ester constituted the major portion of the esterified phosphate. From the monophosphate fraction he was able to isolate trehalosemonophosphate and small amounts of a monophosphate that closely resembled the Robison ester in its properties. Attempts to detect the presence of galactose-phosphate by the methylphenylhydrazine test failed. Perhaps of even greater weight was the fact that he showed that a preparation from adapted yeast, which would ferment galactose, failed to ferment synthetically prepared galactose-6-phosphate. On the basis of this evidence, he concludes that galactose-6-phosphate is not an intermediate in the fermentation of galactose by adapted yeast cells. Of further interest is the evidence he presents that the living yeast cell continues to build up the same polysaccharides when galactose is the sole carbohydrate metabolized as when the carbohydrate is glucose. Hydrolysis of these polysaccharides indicated that they are polymerides chiefly of glucose, and to a lesser extent of mannose and fructose.

Kosterlitz ('43) found that galactose-1-phosphate is fermented by extracts of galactose-adapted yeasts. He found further that, although such extracts fermented glucose at higher rates than galactose, the fermentation rates of galactose-1-phosphate and glucose-1-phosphate were identical. It must be noted, however, that the fermentation rate of the mono-esters was lower than the corresponding

ones for the free hexoses. On the basis of the equality of the fermentation rates of mono-phosphate esters, Kosterlitz postulates the existence of the following equilibrium,

$$\text{Galactose-1-p} \leftrightarrows \text{Glucose-1-p.}$$

However, the same experimental results (unequal rates for the unphosphorylated hexoses, equal but lower rates for the monophosphates) can be explained if either the phosphotase activity or phosphate acceptance were limiting. This would also explain the lower rate of fermentation rate of the esters.

Kosterlitz, on the basis of his experimental results, proposes the following hypothesis of adaptation to galactose fermentation: the formation of two new enzymes is involved. Enzyme (1) phosphorylates galactose at C_1 probably by a system similar to the hexokinase-adenylpyrophosphate systems which phosphorylates glucose and fructose at C_6 (Meyerhof, '35). Enzyme (2) converts galactose-1-phosphate to Robison ester, probably by way of glucose-1-phosphate and subsequent action of phosphoglucomutase or isomerase.

The evidence for the formation of two enzymes stems from the observation that in two out of five samples of dried yeast (Kosterlitz, '43), the yeasts were adapted to ferment glucose-1-phosphate but could not ferment non-phosphorylated galactose.

The bulk of the evidence presented strongly indicates that the adaptive utilization of galactose occurs through the early entrance of the galactose into the glucose metabolic cycle. A non-enzymatic equilibrium (e. g., glucose-1-P $\leftrightarrows$ galactose-1-P) is ruled out by the existence of galactose non-adaptable but glucose-fermenting strains. On the same basis it is clear that the induction period observed in galactose adaptation is not analogous to the lag period observed in glucose fermentation by ordinary yeast brei, which is explained on the basis of a relatively slow accumulation of phosphate esters.

From the studies of the biochemistry of the adapted cell, as well as the stereochemical structure of galactose, it seems most probable that two enzymes at least are formed during the course of the adaptation: One for the phosphorylation of galactose and one for the isomerization of the phosphorylated product into an intermediate of glucose fermentation. In so far as the adaptation is concerned, it would be necessary to postulate that a single substrate can induce the formation of enzymes other than the one for which it is the specific substrate. This, however, raises no real difficulty since, if the enzymes act serially, the product of the first would be the substrate of the second and so on. Thus one substrate could provide a whole series of substrates for a whole series of enzymes.

THE PHYSIOLOGY OF ADAPTATION

In contrast with the relatively vigorous investigation into the biochemistry of the galactose-adapted cell, little has been reported which elucidates the nature of the preadaptive period and the conditions under which the enzyme activity makes its appearance. Certain facts were, however, established by the earlier work.

Attempts to obtain adaptation with non-viable cells or with cells whose physiology has been seriously interferred with by various reagents have uniformly met with failure. The sole exception was the report by Abderhalden ('25) that he had obtained adaptation with dried dead yeast cells. This experiment has never been successfully repeated, and his failure to check the possibility that the adaptation may have been due to the growth of a few surviving cells throws some doubt on the validity of his conclusions. Von Euler and Nilsson ('25) claimed that adaptation will not occur when the cells are suspended in ordinary phosphate containing galactose and they maintained that the addition of "Z" factor was necessary. The earlier experiments of Dienert ('00) contradicted this, since this author did all his experiments with washed cells in ordinary solutions of phosphate.

Our own experience agrees with that of Dienert. All our adaptations were performed with thoroughly washed cells suspended in phosphate solutions of twice-recrystallized galactose. According to these results, the enzyme can be

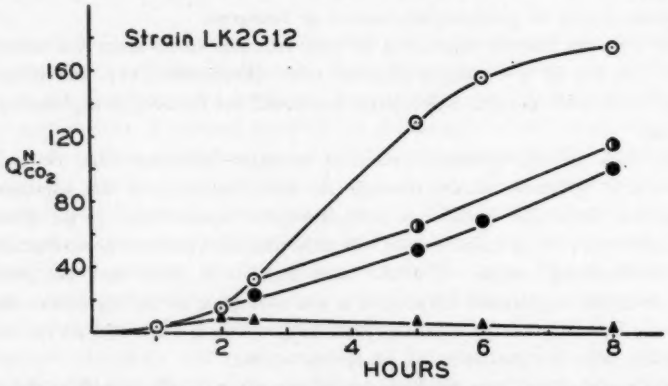


Fig. 2. A test of the ability of cells to increase their galactozymase activity subsequent to aerobic induction periods in contact with galactose. The open circles are the values arrived at for a culture having continuous access to oxygen; the triangles, full-shaded and half-shaded circles represent the subsequent behavior during anaerobiosis.

formed in the absence of an external source of nitrogen. One must conclude, then, that there exists in the cell a source of proteins on which the cell can draw for enzyme formation. It must be noted, however, that the attainable activity level is about half that arrived at if an external source of nitrogen, in the form of ammonium salts or amino acids, is provided (Spiegelman, '45).

Using non-dividing diploid populations, an attempt was made to begin the study of the connection between the synthesis of these enzymes and the over-all metabolism of the cell. Examination of the effect of oxygen (Spiegelman, '45a) revealed that the adaptation was extremely sensitive to oxygen (cf. Stephenson and Yudkin, '36; Schultz, Atkin and Frey, '40). Some strains were found that were completely unable to form galactozymase if they experienced only anaerobic

contact with galactose, while others could form the enzyme anaerobically. However, the rate of anaerobic adaptation was approximately 1/40th of that attained in oxygen. In the case of the strains unable to adapt anaerobically, it was of some interest to determine whether enzyme formation could occur at all under anaerobic conditions. This was done by following the activity anaerobically subsequent to brief periods of aerobic incubation with galactose. The results obtained are given in fig. 2. The open circles are values attained during continuous aerobic incubation with 4 per cent galactose. The anaerobic behaviour of the enzyme activity, following various periods of aerobic contact, is represented by the solid triangles and full and half-shaded circles.

It is seen that at the end of the first hour of aerobic contact, the $Q^N_{CO_2}$ value is about 2.8. Under anaerobic contact there is an initial slight rise above this value and a subsequent slow but consistent drop. At the end of two hours of aerobic contact, the $Q^N_{CO_2}$ attains a value of 15, and subsequent anaerobiosis does not prevent its increase, although the rate of increase is slower than that obtained in the continual presence of oxygen.

It is clear from these experiments that, while adaptation cannot be initiated anaerobically in these strains, it can proceed under these conditions providing an adequate enzyme activity has been built up. The condition to be met here appears to be that enough enzyme be formed aerobically to utilize the energy content of the galactose molecule when anaerobiosis is established at a rate adequate for further synthesis. We have here the interesting physiological situation where the substrate not only stimulates the formation of an enzyme, but, in addition, acts as the only source of energy for its synthesis. That the energy supply is critical seems clear from two facts. It has been shown (Spiegelman and Nozawa, '45) that for these strains, in common with others (see Stier and Stannard, '35a, b), the endogenous reserves are not fermentable. Further, supplying external fermentable substrate (e. g., fructose and, under certain conditions, glucose) permits (Spiegelman, '45b) the adaptation to take place anaerobically in those strains in which it ordinarily does not occur.

These results suggested that the aerobic adaptation occurred because, under these conditions, the cell could draw on the energy coming from the oxidation of the endogenous reserves for synthetic activity. Experiments were therefore performed to examine adaptation times (time to reach a $Q^N_{CO_2}$ value of 100) when the galactose was added at different levels of the endogenous respiration.

The results on one strain are given in fig. 3, in which, for purposes of orientation, the endogenous respiration curve is also diagrammed. It is clear from this figure that up to the zero-rate portion of the endogenous curve little difference in adaptation times is encountered. However, the important point to note is that, although adaptation times increase as the galactose is added further out along the zero-rate portion, nevertheless adaptation occurs. These experiments would seem to indicate that adaptation can take place after all the oxidizable reserves have been exhausted and that there is no apparent source of energy. This

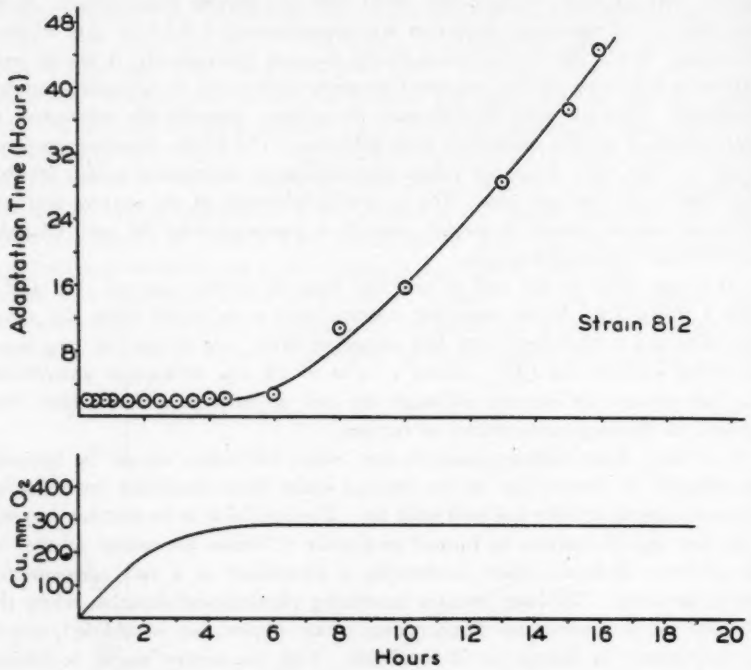


Fig. 3. The effect on adaptation time of adding the galactose at different levels of the endogenous respiration.

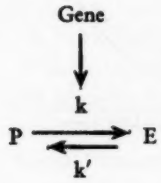
situation was, however, clarified by the finding (Spiegelman, '45b) that the galactose itself is oxidized by some enzyme system other than the fermentative one, which forms later under its stimulation.

It is clear from these experiments that the adaptation is intimately connected with the metabolic activity of the cells. Both aerobic and anaerobic processes are equally capable of supplying the energy for synthesis. Further experiments, which will be detailed elsewhere, indicate that agents which interfere with nitrogen assimilation (e. g., azide) completely suppress adaptation.

GENETIC INFERENCES FROM THE KINETICS OF ADAPTATIONS

It was pointed out in the introduction that, since enzymatic adaptations involved enzyme formation, a careful study of such processes could provide a clue as to the nature of the controls exercised by the genes over the enzyme constitution of cells. The most obvious and easily measured aspect of adaptation is the increase in enzymatic activity observed in non-dividing cells placed in contact with substrate. The usual description of gene action assumes that the gene

mediates directly the reproduction of the enzyme which it controls. From this point of view, every replication of every enzyme would require the intervention of the appropriate gene. On this basis we would ascribe the increase in enzyme activity, observed in the presence of substrate, to the stabilizing influence of substrate on the enzyme. It is proper to inquire what kind of activity time curve such an hypothesis would predict. We may picture the above mechanism by the following reaction diagram.[2]



(1)

Here P is the immediate precursor (perhaps some indifferent protein) whose transformation yields E, the enzyme, the activity of which is being measured. The velocity constant of the transformation from P to E is k, and its magnitude is determined by the gene controlling the reaction. The enzyme E is, however, very unstable and reverts to P quickly, the velocity constant of the back reaction being very much larger than that of the forward one. Under such conditions only very small amounts of E would accumulate in the cell. We now assume that substrate S stabilizes E and that in the presence of excess substrate, ES is formed predominantly. This effectively suppresses the value of k'. In the presence of substrate, the rate of the appearance of enzyme is then described by:

$$\frac{dE}{dt} = k(\overline{P} - E) \qquad (2)$$

where E represents the number of units of P transformed into enzyme in unit time and $\overline{P}$ is the initial amount of precursor present. Integrating and assuming for simplicity that E is zero when t is zero, we find:

$$E = \overline{P} - \overline{P}e^{-kt} \qquad (3)$$

According to equation (3), the assumption that the enzyme is increasing, due to synthesis by the gene and stabilization by substrate, predicts the curve depicted in fig. (4a) as the shape of the activity-time curve during the course of the adaptation. This curve implies that, during the entire period of adaptation, the rate of enzyme synthesis should decrease continuously at any given moment, in a manner proportional to the amount of new enzyme formed at that time. In the

[2] It may be noted that this system is a logical analogue of Yudkin's ('38) "mass action" theory of enzyme synthesis.

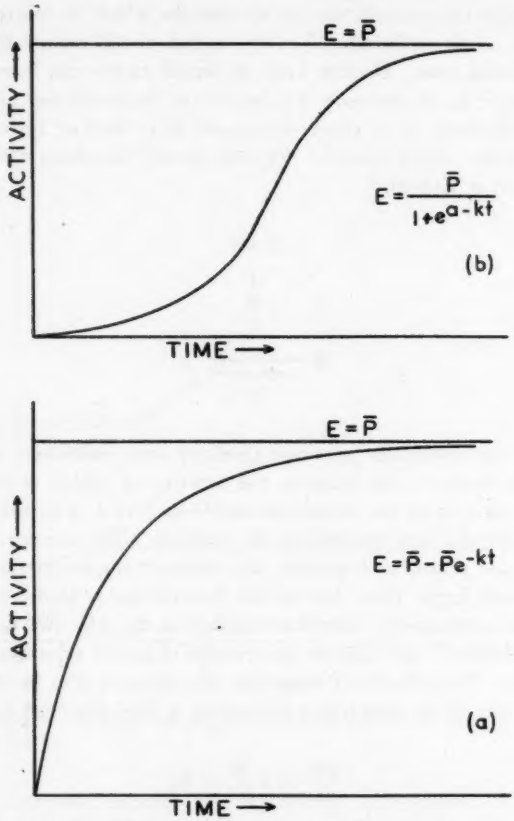
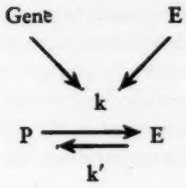


Fig. 4. Activity-time curves predicted by (a) direct primary gene control of enzyme synthesis and (b) self-duplication of enzyme molecules.

course of examining the synthesis of the glucozymase and melibiozymase systems, over 400 adaptation curves have been obtained. In no case does the activity-time curve resemble the course predicted by the above analysis. In all instances (see e. g. fig. 2, open circles) the initial part of the curve is characterized by a rising rate of enzyme formation. This is then followed by a declining rate portion, when presumably the indifferent substrate becomes limiting and finally exhausted.

The increasing rate of enzyme synthesis, with increasing amount of enzyme, suggested an obvious modification of the mechanism detailed in diagram (1). Retaining all the properties ascribed to the first mechanism, we add the additional one that the enzyme once formed can duplicate itself without further need for genic intervention. With this self-duplication hypothesis, instead of reaction diagram (1), we have,



(4)

where the symbols have the same meaning. As before, we suppose that the gene can transform *P* into active enzyme *E*, which is unstable in the absence of substrate. The arrow going from *E* to *k* symbolizes the self-duplication of the enzyme, which would express itself in terms of changing velocity constant so that its value at any particular moment would depend on the amount of *E* present. In the presence of substrate, the rate of formation of the enzyme becomes, under these assumptions, a quadratic function of the amount of enzyme present and takes the form:

$$\frac{dE}{dt} = kE(\bar{P} - E) \qquad (5)$$

where *E* again represents the amount of *P* transformed into *E* in unit time and $\bar{P}$ is the initial amount of precursor present. Integrating equation (5) we obtain

$$E = \frac{\bar{P}}{1 + e^{a - kt}} \qquad (6)$$

where *a* is an integration constant determined by initial conditions. According to equation (5) then, the assumption of self-duplication predicts the s-shaped curve given in fig. 4b as the activity-time curve during adaptation.

There is no doubt that the data lend support to the self-duplication hypothesis and rule out the simple genic mechanism underlying reaction diagram (1). More rigorous mathematical and experimental tests have been made and will be detailed elsewhere. An important and critical prediction stemming from the self-duplication mechanism is that, once the process is started, it can proceed in the absence of the gene which initiated it. Attempts to test this prediction were made with data on the inheritance of melibiozymase. The Mendelian mechanism underlying the inheritance of the ability to form melibiozymase was analyzed with the aid of two strains differing in this character.

THE GENETICS OF THE ABILITY TO FORM MELIBIOZYMASE

Hybrids between melibiose-fermenters and non-fermenters had already been examined by Winge and Laustsen ('39a). All hybrids of such crosses were fer-

menters, and their results led Winge and Laustsen to state that "the presence of a specific enzyme is dominant to its absence in all the instances studied." They made matings by placing two spores in contact with each other. This method has the disadvantage that one cannot characterize the haplophase parents, since both of the original spores are consumed in the mating. This, and the failure to examine the phenotypes of the segregants from the hybrids, prevented an analysis of the genetic mechanism.

This analysis was undertaken by Lindegren, Spiegelman and Lindegren ('44), using *S. carlsbergensis*, which could adapt to ferment melibiose, and *S. cerevisiae*, which could not. In this investigation hybrids were produced by mixing haplophase cultures. Since only part of the culture is needed for the mating, the remainder could be used to determine the characteristics of the parent strain, as well as for back-crossing or mating to other clones of interest. All hybrids formed were allowed to sporulate and the asci dissected to permit examination of the phenotypes of the haploid segregants.

The data obtained from 175 progenies of the interspecific and of related hybrids were consistent with the view that *S. carlsbergensis* contains two pairs of dominant genes (mel+), either one of which permitted the production of melibiozymase. Since all of the haploid segregants of *S. cerevisiae* failed to produce the enzyme, it was clear that it was homozygous for the recessive alleles.

SELF-DUPLICATION OF MELIBIOZYMASE IN THE ABSENCE OF ITS GENE

With the genetics of the capacity to form melibiozymase known, it became possible to devise experiments which would test the self-duplicating hypothesis suggested by the S-shaped adaptation curves. In particular, it was essential to provide answers to the following questions:

(1) If synthesis has been initiated and the gene's allele substituted by segregation, can the substrate-cytoplasmic interaction maintain the enzyme indefinitely in the cytoplasm in the absence of the specific gene?

(2) If some enzyme is present, can synthesis of additional enzyme occur in the absence of the specific gene necessary to initiate its synthesis?

Use was made of progenies of known genetic composition from the *S. cerevisiae* x *S. carlsbergensis* pedigree employed in the study of the Mendelian mechanism of melibiozymase inheritance. In the experiments described in the previous section, the cells came into contact with melibiose for the first time in the test for adaptability after segregation had already taken place. To answer the questions posed above, experiments were performed (Spiegelman, Lindegren and Lindegren, '45), in which the matings as well as the segregations were carried out in the presence of melibiose. The results were compared with matings from the same cross in which melibiose was omitted until testing the phenotype of the haploid segregants. To simplify the genetics of the situation, a haplophase clone carrying a single mel+ gene controlling adaptation was used. This was mated to a haplophase clone of *S. cerevisiae* which carried only the recessive alleles. The heterozy-

gous diploids so formed were all adaptable and each four-spored ascus from these hybrids yielded two adaptable and two unadaptable haplophase cultures.

TABLE II

EFFECT OF MELIBIOSE ON PHENOTYPIC CHARACTERS OF SEGREGANTS FROM DIPLOIDS FORMED BY MATINGS IN ITS PRESENCE AND ABSENCE

Mating, sporulation and planting in presence of melibiose					Mating, sporulation and planting in absence of melibiose				
Ascus No.	Spores* A	B	C	D	Ascus No.	Spores* A	B	C	D
1	+	+	+	+	8	+	+	−	−
2	+	+	+	+	9	−	+	−	+
3	+	+	+	+	10	+	+	−	−
4	+	+	+	+	11	+	+	−	−
5	+	+	+	+	12	−	+	−	+
6	+	+	+	+	13	+	−	−	+
7	+	+	−	−	14	+	+	−	−
					15	+	+	−	−
					16	+	+	−	−
					17	−	−	+	+

* + indicates ability to ferment melibiose, — inability. All spores come from a (+ x —) cross. See text for further details.

The data obtained on the phenotypes of the haploid segregants from diploids formed and segregated in the presence and absence of melibiose are summarized in Table II. Asci 1–15 originated from the mating of the same pair of mel+/mel— haploids, while 16 and 17 originated from mating an equivalent, but not identical, pair of mel+/mel— haploids. Melibiose was present during all stages of the formation and dissection of asci 1–7 inclusive. Asci 10–17 inclusive were formed in the usual way, without melibiose. In handling asci 8 and 9, the agar in which the dissected spores were planted contained melibiose.

It is evident from Table II that all asci formed in the complete absence of melibiose give the typical 1:1 ratio characteristic of a heterozygous hybrid segregating a single pair of genes. On the other hand, with the exception of ascus No. 7, identical heterozygotes treated with melibiose yielded four adaptable spores from each ascus.

The results obtained in the absence of melibiose prove that only 2 spores from each tetrad in asci 1–6 inclusive contain the specific mel+ gene responsible for

adaptation to fermentation. Despite this, all four spores from these tetrads produced haplophase cultures which fermented melibiose.

Since all steps were carried out in the presence of melibiose, selection of adaptable mutants from haploids originally unable to ferment melibiose might have occurred. Several specific facts, however, rule out this possibility: (1) During the testing of many haploid segregants from *S. cerevisiae*, all of which are negative, no mutation to an adaptable type has ever been observed whether melibiose was present or not; (2) the same is true of negative haploids from heterozygous hybrids. No mutation to adaptables in these have been seen no matter how often they have been through melibiose media; (3) asci 8 and 9, whose segregants were planted on melibiose, yielded the standard 1:1 ratio.

Presumably, the cultures from the two spores of each tetrad from the first six asci were able to ferment melibiose only due to the presence of the enzyme in the cytoplasm. On this basis it was to be expected that removal of the melibiose would lead not only to the disappearance of fermentability in all cases, but to an eventual loss of readaptability in two of every four cultures arising from each of the first six asci. To exclude the complication of mutation away from adaptability, non-dividing cultures, suspended in M/15 KH_2PO_4, were used. Portions of all 24 adapted haplophase cultures originating from the first 6 asci were dissimilated in the absence of substrate until they had lost all melibiozymase activity. Samples were then removed and incubated with melibiose to test for readaptability. Not all haplophase cultures survived this relatively vigorous treatment which in some cases lasted 20 days. Table III summarizes the results obtained with those asci, all four of whose segregants stood the treatment. The removal of the melibiose and its stabilizing influence leads to the disappearance of the enzyme in the cytoplasm and the reappearance of the expected Mendelian ratios.

Data collected at the same time indicate that synthesis of additional enzyme can occur in the absence of the mel+ gene. After allowing all suspensions to

TABLE III

READAPTABILITY OF SPORES OBTAINED BY MATINGS IN PRESENCE OF MELIBIOSE AFTER HAVING LOST ALL ADAPTIVE ENZYMES

Ascus No.	Spores* A	B	C	D
1	+	−	+	−
2	−	−	+	+
4	+	+	−	−
6	+	+	−	−

* + indicates readaptability, − inability.

fall to low $Q^N_{CO_2}$ values (between 1.8 and 10.1) in the absence of melibiose, portions were removed and incubated with melibiose and regeneration of activity followed at intervals by measuring $Q^N_{CO_2}$. The results of those haploid segregants which subsequently lost the ability to adapt are recorded in Table IV. It is seen that in all cases marked increases in activity were obtained. Furthermore, all the strains listed in Table IV were carried in standard media with melibiose and were tested at weekly intervals. At the end of three months they could all ferment melibiose at rates equal to, or greater than, the original rate. This period is equivalent to over 2,000 cell generations. It is evident that the enzyme can not only maintain itself in the presence of melibiose but it can also increase in absolute amount.

TABLE IV

$Q^N_{CO_2}$ VALUES AFTER AEROBIC INCUBATION WITH MELIBIOSE OF STRAINS WHICH EVENTUALLY LOST THEIR ABILITY TO ADAPT

Strain	Hours of contact with melibiose			
	0	12	24	48
1B	5.1	40	96	123
1D	2.4	26	109	114
2A	10.1	39	86	136
2B	6.3	46	73	101
4C	5.0	69	160	170
4D	4.2	29	91	134
6C	1.8	34	84	141
6D	4.8	42	121	130

The simplest explanation which can be offered at present for the above results is the same one advanced for the S-shaped curve, i. e., the effect of substrate on a self-duplicating enzyme. We may thus explain the effects of melibiose on the inheritance of melibiozymase as follows: by performing the mating in the presence of melibiose, the cytoplasm of the haploid carrying the mel+ gene is packed with the melibiose-fermenting enzyme. Since both copulating haploids contribute cytoplasm equally to the zygote it starts out with some enzyme and builds up more since it has the gene also. Since sporulation occurs in the presence of melibiose and since the sporulation period is characterized by growth and considerable storage, the enzyme molecules are stabilized and possibly increased in amount. Each of the four haploid segregants derives its cytoplasm from the diploid hybrid, and it follows that each will have enzyme molecules in its cytoplasm no matter

what its genetic constitution. Finally, the enzyme molecules are stabilized and duplicate themselves in the descendants of the spores which do not have the mel+ gene as long as they are kept in contact with the substrate.

It cannot be denied that explanations of these results involving unstable genes can be devised. It is further recognized that no analysis of the adaptation curves, no matter how rigorously it is formulated and subsequently tested, can ever prove the self-duplication of enzymes. It is impossible to exclude in any finite period all the conceivable modifications which can be advanced containing as their primary postulate the gene as the sole self-duplicating unit in cells. This much, however, may be said in view of the experiments on melibiozymase and galactozymase—such theorizations will not be "pleasingly simple."

We may, therefore, on the basis of the available evidence, suggest that some enzymes are capable of duplicating themselves without genic intervention. In these cases of enzyme formation, the sole function assignable to the gene is the initiation of the enzyme synthesis. This initiation could be effected by virtue of a low but ever-present capacity of the gene to mediate the production of a few enzyme molecules. A mechanism of this kind would keep some enzyme molecules always available in the cytoplasm for autosynthetic activity when substrate is supplied. It would, at the same time, explain the observed Mendelian inheritance of adaptability in the absence of substrate, as well as the ability of substrate when present to obscure the Mendelian ratios during segregation. In addition, it would provide a rational basis for the S-shaped adaptive curves.

The question of how generally the above concept can be applied cannot be decided without further experiments on other enzyme systems. From a casual observation it might seem that self-duplication of the enzymes is inconsistent with the results on dosage effects. It must, however, be noted that in such studies, end products of enzyme activity, rather than enzymes themselves, are being studied. Furthermore, in order for the self-replication capacity of enzymes to express itself, the precursors would have to be initially present in sufficient quantities. If the enzyme precursor is limiting, the effect of E on the forward velocity constants would be negligible, and diagram (4) would transform to diagram (1). Then, even if the enzyme was able to duplicate itself, the synthesis would be predominantly gene-controlled. The addition of another gene would, under such conditions, effect the quantitative level of enzyme activity. To this must be added, in so far as gene-dosage studies are concerned, possible limitations of the precursor to the end product. In this connection, it is of interest to note that competitive interaction for a limited amount of substrate has been assumed in Dr. Stern's theorizations on the nature of gene action.

Finally, it must be noted, from a general physiological point of view, that self-duplication of enzymes provides a degree of physiological flexibility not easily attained with the older, more rigid concepts of gene control over enzymatic constitution. Under this concept the enzymatic composition is dependent not only upon the genome, but also on the available substrates. Enzymes could thus

increase and decrease in response to the substances placed in their environment. In addition, it provides an experimentally interesting mechanism for cytoplasmic differentiation, since it predicts that cells with the identical genomes need not possess identical enzymatic constitutions.

SUMMARY

The general problem of enzymatic adaptation is discussed. The view is adopted that adaptive-enzyme formation is a quantitatively exaggerated instance of a more general phenomenon involving the effects of substrates on the synthesis and stability of their enzymes. Experiments on the genetic control of adaptation to the fermentation of galactose and melibiose by yeasts are described and discussed in detail. It is pointed out that these instances, where enzyme formation can be followed with relative ease, offer a unique opportunity for examining the often-repeated concept that genes determine phenotype by virtue of their control of the enzymatic constitution of cells. In particular, the extent and mechanism of such control might thus be open to experimental analysis.

A study of the kinetics of the formation of galactozymase and melibiozymase in yeast cells is detailed which suggested that at least in these cases the enzymes were capable of self-duplication without the necessity of genic intervention. The hypothesis of self-duplication led to the prediction that such enzymes, once formed, should maintain themselves and be transferable from one cell generation to the next in the complete absence of their corresponding genes. Experiments on the inheritance of melibiozymase in the presence and absence of melibiose are reported which tend to confirm this prediction. It is suggested that, in these cases of enzyme formation, the sole function assignable to the gene is the initiation of the enzyme synthesis, this initiation being effected by virtue of a low but ever-present capacity of the gene to mediate the reproduction of a few enzyme molecules. Subsequent replication is dominated by the self-duplicating enzyme molecules.

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THE MECHANISM OF RADIATION EFFECTS AND THE USE OF RADIATION FOR THE PRODUCTION OF MUTATIONS WITH IMPROVED FERMENTATION

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PART I

In discussions of the effects of radiation, the ultraviolet spectrum is usually divided into the so-called biologically effective region between 2000 and 3200Å and the non-active region longer than 3300Å. Studies conducted during the last few years have shown that both of these regions of the spectrum are effective. However, the energies necessary to produce recognizable effects are of a different order of magnitude. The modes of action of the various wavelengths of the ultraviolet are fundamentally different, apparently affecting various structures of the cell.

The region shorter than 3200Å is characterized by its high absorption by proteins and nucleic acids, the proteins by their low absorption band in the 2800Å region and high absorption at wavelengths shorter than 2300Å; the nucleic acids by their extremely high absorption band at 2600Å. In general, absorption spectra of biological material will show a pattern resembling protein absorption or show slight modification usually indicating nucleoproteins. It is only when the nucleic acid is concentrated in certain structures as, for instance, chromosomes, that its location can be readily recognized as has been shown by Caspersson ('36).

Considerable information in regard to the chemical characterization of the biological effect of radiation can be obtained from wavelength dependence studies of biological effects. Another method for determining what radiation will do to the cell is to extract its chemical constituents and follow their change *in vitro* by certain physical and chemical techniques. A further method is to follow changes in certain morphological structures produced by specific wavelengths in living cells. We have used all three approaches in our studies. However, we have obtained the most extensive data by the first method which I have mentioned, and rather fragmentary data by the other approaches. In studying the effects of radiation on biological materials, we have concentrated our efforts on problems which would be of direct or indirect significance to public health. This, of course, is not very difficult, since any fundamental biological approach will help us with the interpretation of the relation of disease to health.

I will discuss first a typical wavelength dependence study which we have recently completed (Hollaender and Oliphant, '44). The sensitivity of influenza virus A was determined for 8 wavelengths in the ultraviolet spectrum between 2180 and 2967Å. To get a definite measure of the sensitivity of this virus, we have irradiated a standard culture of *Escherichia coli* in the virus suspension. The

sensitivity of this organism to monochromatic radiation is well established. We found only a slight difference in the resistance of *Escherichia coli* and influenza virus. There is also very little difference in wavelength dependence of their inactivation. The absorption spectrum of influenza virus, as well as of bacteria, shows predominantly the type that you would expect from proteins mixed with a small percentage of nucleoproteins, whereas the inactivation spectra resemble more closely the pure nucleic acid absorption spectrum.

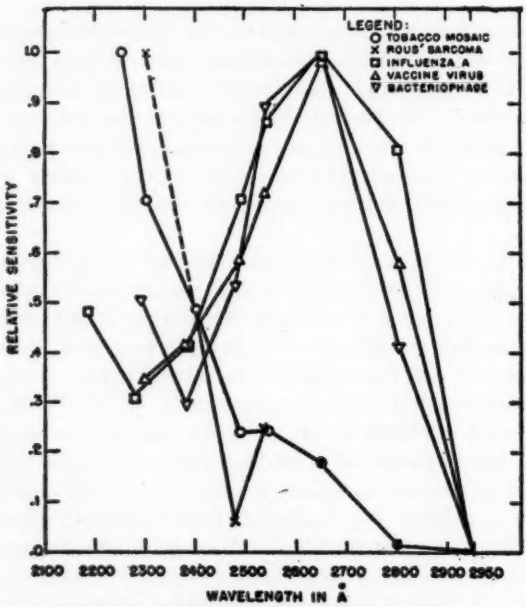

Fig. 1. Plot of the relative sensitivity against the wavelength for tobacco mosaic, Rous' sarcoma, influenza A, vaccine virus and bacteriophage, taking the energy at the wavelength which is most effective as 1 and dividing by the less effective energies. (For references see Table I, Hollaender and Oliphant, '44.)

Do all the viruses behave the same way? There are available activity spectra for five viruses: three of these have a maximum of sensitivity at 2650Å and decreasing sensitivity at shorter or longer wavelengths, and two which have a high sensitivity at 2300Å and considerably lower sensitivity in the 2600Å range. Influenza A, vaccine virus, and bacteriophage belong to the first group, and the viruses of typical tobacco mosaic and of Rous' sarcoma belong to the latter group. I mentioned before that the absorption spectra of viruses resemble more closely protein spectra with a small band typical for nucleic acids. There seems to be little doubt that, on the basis of these findings, the nucleic-acid part of the influenza, vaccine virus, and bacteriophage is the most sensitive part of these

particles, and that the protein part of tobacco mosaic and Rous' sarcoma virus is the least resistant one.

Another point which should be emphasized is that the nucleic acid of influenza, vaccine virus, and bacteriophage has been reported to be desoxypentose and the nucleic acid of tobacco mosaic and Rous' sarcoma is predominantly pentose. (For details of this study see Hollaender and Oliphant, '44. See later discussion of irradiation of nucleic acids *in vitro*.)

TABLE I

REACTIONS WITH HIGHEST SENSITIVITY AT 2650A

Inactivation of viruses and virus-like agents		Influenza (Hollaender & Oliphant, '44) Vaccine (Rivers & Gates, '28) Bacteriophage (*St. aureus*) (Gates, '34)
Killing of microorganisms	Bacteria	All types—Pathogenic and Saprophytes (For review see Hollaender, '42)
	Fungi	Yeasts (Oster, '34) *Trichophyton* (Hollaender & Emmons, '39) *Aspergillus terreus* (Hollaender, Raper & Coghill, '45)
Mutation production	Fungi	*Trichophyton* (Emmons & Hollaender, '39) *Neurospora* (Hollaender, Sansome, Zimmer & Demerec, '45) *Aspergillus terreus* (Raper, Coghill & Hollaender, '45) *Penicillium notatum* (Hollaender & Zimmer, '45)
	Higher Organisms	*Sphaerocarpus* (Knapp, Reuss, Risse & Schreiber, '39) *Zea Mays* (Stadler & Uber, '42) *Drosophila*: MacKenzie & Muller, '40; Demerec, Hollaender, Houlahan & Bishop, '42
REACTIONS WITH VERY HIGH SENSITIVITY AT λ 2300A AND A SMALL MAXIMUM AT 2800A OR 2600A		
Inactivation of viruses and enzymes		Tobacco mosaic (Hollaender & Duggar, '36) Rous' sarcoma (Sturm, Gates & Murphy, '32) Urease (Kubowitz & Haas, '33)
Killing of higher organisms		*Enterobius vermicularis* (Hollaender, Jones & Jacobs, '40) *Ascaris* (Wright & MacAlister, '34)
Parthenogenesis of *Arbacia*		*Arbacia* (Hollaender, '38)

Table I shows a list of biological reactions for which sufficient data are available on the effect of monochromatic ultraviolet radiation to permit their being

classified and fitted into the predominantly protein or the nucleic acid pattern. It is not surprising that bacteria have a maximum sensitivity at 2650Å, since, as far as tested, they are made up of a high percentage of nucleic acids. The same applies to yeasts and fungi but the 2600Å maximum of sensitivity in many fungi is obscured by the protective absorption of pigments.

Whenever 2600Å radiation has been tested for mutation production and the conditions have been such that the radiation could penetrate readily to the nucleus, high efficiency in producing genetical changes for this wavelength has been found. Our early studies with Dr. Emmons on *Trichophyton* have now been repeated with *Neurospora* in a cooperative study with Dr. Demerec and Mrs. Sansome. The results of this study verify our findings on *Trichophyton:* 2650Å is the most efficient wavelength in producing mutations. This work on fungi, which I will discuss in the second part of my paper, has also been extended to *Penicillium notatum* and *Aspergillus terreus.*

I am sure you are acquainted with the work on higher organisms. I included the work on *Drosophila* under the 2650Å section in spite of the fact that the wavelength which is most effective on this organism is 3130Å. The probable reason for this is that the sperm has to be irradiated inside the fly and the abdominal wall prevents the 2650 wavelength from penetrating readily to the sperm. It is unfortunate that the artificial insemination technique has not proved practical.

The second part of this table shows a number of biological reactions with high sensitivity in the very short ultraviolet (<2300Å). This would indicate that the protein part of these materials is the most sensitive one. The inactivation of tobacco mosaic and Rous' sarcoma shows also a small maximum at λ 2600Å, indicating that the nucleic-acid part of these materials has a slight sensitivity in this region.

The higher organisms described in the rest of this table are surrounded by heavy protein membranes which explain their sensitivity in the short ultraviolet. Little information is available at the present time in regard to tissue cultures. Crude work has shown that this material shows its highest sensitivity at short wavelengths.

It would not be surprising, however, that careful studies which take into account the action of protective materials would bring out a fairly high sensitivity in the 2600Å region.

Summarizing, it is well to point out that the wavelength-dependence studies have given us an opportunity to obtain an indication of the chemical structure in living substance, which is most easily interfered with by radiation.

In an effort to get a better understanding of the effect of radiation on living materials, we studied some of the constituents of living cells *in vitro.* We have studied the effect of 2537Å radiation on sodium thymonucleate (Hollaender, Greenstein and Jenrette, '41) and certain serum proteins (Davis, Hollaender and Greenstein, '41). The changes most readily produced are the result of alteration in the

physical properties of the treated compounds, for example: viscosity, stream birefringence and colloid osmotic pressure. While the changes produced in the isolated compounds of the living cell or directly in the living cell are doubtless qualitatively similar, quantitatively they must appear to differ enormously. This is probably due to the difference in detectability of the two types. Changes in the isolated components must be detected by physical methods which require that a relatively large number of the molecules of the compounds under study be altered, and this, in turn, requires very large doses of radiation. The structures of the living cell, on the other hand, even though they consist of these same or similar compounds are parts of very delicately balanced and precisely adjusted units, in which changes induced in a few molecules by relatively low doses of radiation may alter radically certain detectable behavior and structural characteristics of the cell (Carlson and Hollaender, '44). Very little is known about the state of the relation of protein to nucleic acids in living cells. A search of the literature on this subject reveals that there is still considerable confusion about the exact structure of nucleo proteins (Greenstein, '44) and further work in this field is urgently needed.

The effect of 2537Å was studied (Carlson and Hollaender, '44; Kaufman, Gay and Hollaender, '44) in an effort to obtain information in regard to the mechanism of the influence of radiation on mitosis. Although this study is in its early phases, the results indicate that the early prophase is retarded most by 2537Å radiation, in contrast to X-rays where the middle and late prophases are most sensitive. The high sensitivity of chromosomes to 2537Å radiation is well demonstrated by the fact that an exposure to a total of 1500 ergs per square centimeter, either given in 1 second or spread over 1500 seconds, will produce a measurable retardation of mitosis in grasshopper neuroblasts in tissue cultures.

Up to this point, I have discussed the effects of radiation shorter than 3200Å. The action of radiation in the long ultraviolet has been more or less ignored. One reason is that most non-pigmented biological materials have very little absorption in this region; as a matter of fact, so little that our present means of taking absorption spectra are not sensitive enough to detect this absorption. This also explains why the energies necessary to produce changes in the long ultraviolet are of different order of magnitude than the ones at shorter wavelengths. For instance, we can produce very striking effects if we give bacteria 10,000 to 100,000 as much energy at 3650Å as was necessary to produce recognizable effect at 2650Å. Besides its lethal action, 2650Å will produce a delay of growth in surviving organisms; in other words, a prolongation of the "lag" phases. This prolongation of the lag will be about 50 per cent of the normal lag. The 3650Å range may increase the normal lag phase tenfold. It will also change the permeability of the cell. We have summarized these effects in Table II.

It appears that the effect produced by this wavelength is through action on the colloid structure of materials irradiated as well as in the structure of certain respiratory enzymes. The function of the long ultraviolet is important from

TABLE II

EFFECTS OF LONG ULTRAVIOLET AND NEAR VISIBLE RADIATION ON *ESCHERICHIA COLI*

	3400 to 4400Å	2180 to 2967Å
1. Shape of killing curve (log survival ratio/energy)	Threshold type	Approaching straight line
2. Energy (incident) for 50% survival ratio	Approximately 2×10^5 ergs/cm.^{2}	5×10^2 to 10^3 ergs/cm.^{2}
3. Temperature coefficient	1.7 — 2.2	1.1
4. Sublethal effects appear	Before any organisms are killed (in threshold part of killing curve)	After 60 to 90% of organisms are killed
5. Extension of retarded growth phase for 10% survival ratio	Up to 1000%	50%
6. Toxicity of certain salt solutions can be recognized	At once after irradiation	In 600 minutes at 32° C.
7. Mutation production	No mutations	Mutations produced in fungi and *Drosophila*

the ecological point of view, since this radiation is quite intense in sunlight.

In summary, the study of the response of microorganisms to ultraviolet radiation has established distinct effects which each wavelength range produces. The wavelengths which are most highly absorbed by nucleic acids (2600Å) are most efficient in producing mutations. Other wavelengths which are absorbed more generally by the cell (3650Å) show their effect in a retardation of growth and an interference with the normal respiration of the cell. Several regions of the spectrum still await an interpretation of their effects on the living cells. The field of the combination of different wavelength ranges is an especially promising one for further investigation.

PART II

The production of mutations by ultraviolet radiation follows a definite quantitative pattern. The maximum mutation rate is reached after the organisms have been exposed to certain amounts of energy. A further increase in energy tends to decrease the mutation rate from this maximum rate. In contrast to this, the increase of mutation rate with increasing energy in the X-ray region is more or less linear. These typical mutation curves have been established not only with the *Fungi Imperfecti* but have also been found with *Neurospora crassa* (Sansome, Demerec and Hollaender, '45; Hollaender, Sansome, Zimmer and Demerec, '45).

It was thought when this work was begun that it might be possible, by radiation techniques alone, to produce mutations of certain predetermined properties. Experience has shown that it is not yet possible to accomplish this. However, it has been found that the ultraviolet will produce a predominance of gene mutations while the X-rays tend to produce a predominance of chromosomal aberrations and chromosome breaks (Stadler and Uber, '42).

Most of the early work on mutation production in fungi established the mutation rate on the basis of "morphological" changes. But the fundamental reactions which cause the appearance of morphological mutations are no doubt "biochemical." Early in the war it became desirable to produce changes in certain organisms which were capable of producing urgently needed chemicals. This led us to suggest the use of radiation techniques for this purpose.

The usual tendency in all induced mutation work is to produce changes in the organisms which result in reduced activity. This is probably due to an interference with certain enzyme systems. This type of approach has been established by Beadle and Tatum ('41). The so-called "progressive mutations," i. e., mutations with improved fermentation, have only occurred occasionally. The difficulty here probably lies in the fact that several gene modifications are necessary to induce a mutation with increased yield while a "deficient" mutation may be caused by single gene changes.

The results of most fermentation processes of fungi are not alcohols, acids, etc., of high purity, but usually a mixture of more or less closely related compounds. Thus the suppression of an undesirable reaction, through interference with the enzyme system causing it, is a promising possibility. However, as can be seen below, if one interferes with one enzyme system, there is a tendency for the whole chain of systems to be disrupted, probably because of the close interrelationship of the different systems within the organism.

We will now discuss a number of studies where an attempt has been made to influence fermentation in a direction most desirable to the experimenter. Cultures which survived X-radiation and show deficiencies in development were observed as early as 1904 at a time when the early biological exploration of Roentgen's discovery was at its height (Dauphin, '04). Observations of Nadson ('25) showed that colonies of yeast on the border of irradiated areas in Petri dishes grow more profusely than the colonies protected against radiation.

An extensive study of the production of citric acid by *Aspergillus niger* as influenced by radium emanation and ultraviolet light was reported by Kresling and Stern in 1936. They observed an increase of citric acid in the cultures when grown in the presence of radon, but no increase of citric acid was observed when the cultures were grown under ultraviolet. A number of strains were isolated from the irradiated cultures. The results of these tests for acid production of these mutations are given in Table III. All of the new strains produce equal or less amounts of acid than the controls.

Early in the development of the mass production of penicillin by *Penicillium*

TABLE III

BIOCHEMICAL PROPERTIES OF RADIUM STRAINS OF *ASPERGILLUS NIGER**

Strains	Acid in grams per 100 ml fermentation solution			Sugar in grams per 100 ml solution		Dry weight of mycelium in grams
	Citric Acid	Gluconic Acid	Oxalic Acid	Used	Left over	
Control Strain #3	5.21	0.90	0.19	13.35	6.65	4.579
Radium Strain #3_1	0.18	2.91	0.00	11.66	8.34	3.822
Acid Strain #3_2	0.00	2.60	0.00	12.50	7.50	4.132
Control Strain #1	1.64	0.34	0.33	18.7	1.3	4.934
Radium Strain #1_1	0.00	0.58	0.00	15.0	5.0	4.872
Control Strain #6	11.88	1.16	0.33	18.7	1.3	3.554
Radium Strain #6_1	0.68	0.84	0.33	15.0	5.0	3.869
Radium Strain #6_2	0.00	2.33	0.20	14.0	6.0	4.761
Radium Strain #6_3	7.52	0.73	1.00	15.4	4.6	3.624

*Table taken from: Über die Wirkung von Radium- und ultravioletten Strahlen auf die Entwicklung, die biochemischen Eigenschaften und die Rassenbildung des *Aspergillus niger*. (Kresling and Stern, '36, p. 339).

TABLE IV

EXPERIMENT F3—*PENICILLIUM NOTATUM*. CULTURE 10 DAYS OLD IRRADIATED WITH λ 2650A

	Energy per spore	Per cent survival	Number colonies isolated	Per cent mutation
Control	0	100	75	0
Run 1	3.0×10^{-3} ergs	43.0	74	1.3
2	9.4	37.4	76	1.3
3	19.5	14.7	75	2.0
4	34.1	5.4	81	13.6
5	42.9	.2	81	12.3
6	71.5	.03	80	8.8

notatum, we had an opportunity to discuss with Dr. Heatley of the Oxford group the possibility of producing mutations with increased penicillin yield by irradiating *Penicillium notatum* spores with monochromatic ultraviolet and possibly X-rays. We started this investigation in cooperation with Dr. Emmons of the National Institute of Health in 1941 and later continued it in cooperation with the Cold Spring Harbor group.

The production of the morphological mutations follows the usual pattern: high efficiency of mutation production with wavelength 2650, lower efficiency at shorter and longer wavelengths, and no mutation production at wavelengths in the 3650Å region. The mutation rate increased with increasing energy and became more or less erratic at still higher energy values. In contrast to this, the effect of X-rays on morphological mutations follows a more or less straight-line relationship. I will return to this point later on. Typical results of a single irradiation test are given in Table IV. A typical killing and a mutation curve are shown in fig. 2.

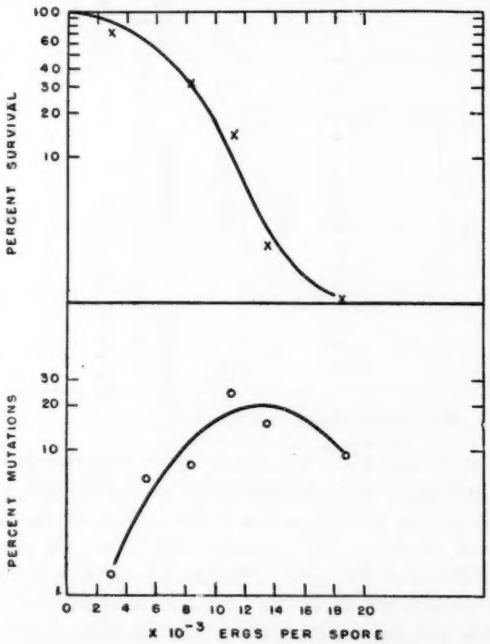

Fig. 2. *Upper graph:* Per cent survival against energy absorbed per spore for *Penicillium notatum*. *Lower graph:* Per cent mutation against energy. Each point on the lower graph corresponds to point in upper graph for same energy value.

No general biochemical investigation was conducted in connection with this study. However, in a collaborative study with Dr. J. W. Foster of Merck & Co.,

at Rahway, N. J., a number of irradiated and control cultures were tested for penicillin production at the Rahway laboratory. Penicillin production was determined after 2, 3, 4, and 5 days in submerged shaking cultures. The concentration was determined by the standard cup method. The results of a typical set of data are given in Table V. Typical yield distribution plots are given in fig. 3. The mean of the penicillin production for control and irradiated cultures is also given.

TABLE V

TYPICAL SET OF TESTS FOR CULTURES OF *PENICILLIUM NOTATUM* COMING FROM IRRADIATED AND CONTROL SPORES (JULY 1943)*

Culture	Oxford Units per ml		
	2 days	3 days	4 days
F_2 5.25	17	25	8
26	23	29	11
27	18	40	13
28	17	43	28
29	24	52	29
31	26	42	28
32	19	40	29
33	< 8	< 8	29
36	< 8	< 8	28
F_3 5.10	< 8	< 8	8
13	< 8	< 8	8
32	< 8	11	13
36	< 8	54	8
37	< 8	22	8
44	22	80	8
45	28	49	11
49	23	54	8
56	16	56	12
60	25	59	20
66	24	80	32
Control	33	46	17

*Tested by J. W. Foster, Merck & Co., Rahway, N. J.

The irradiated cultures show, in general, a very wide distribution of variation in the yield of penicillin with a predominance of low-yielding strains and some which practically did not produce any penicillin. However, occasionally a mutation was produced which gave an unusually high yield. Of about 200 cultures tested, two were found of this type. The distribution of yield of cultures seems to be definitely towards the lower side. It is unfortunate that the difficulty of testing *Penicillium notatum* for penicillin production makes it cumbersome to run through a large number of cultures under a variety of conditions which might bring out more clearly the interesting mutations. There seems to be little, if any, relation between morphological mutation and change in penicillin production. As a matter of fact, the normal-appearing cultures have a tendency to give the higher yields.

Another study was conducted in cooperation with Dr. Raper, Dr. Coghill and

others of the Northern Regional Laboratory (Hollaender, Raper and Coghill, '45; Raper, Coghill and Hollaender, '45). The purpose of this investigation was to attempt to produce mutations in *Aspergillus terreus* which would have increased itaconic acid production.

This organism distinguished itself by a very low sensitivity to ultraviolet radiation. However, it showed itself to be more sensitive to mutation production than any of the other organisms tested. While most of the organisms tested show their highest mutation rates with ultraviolet when 90 to 95 per cent of spores are killed, *Aspergillus terreus* shows its highest mutation rate after 25 to 40 per cent of the spores are inactivated.

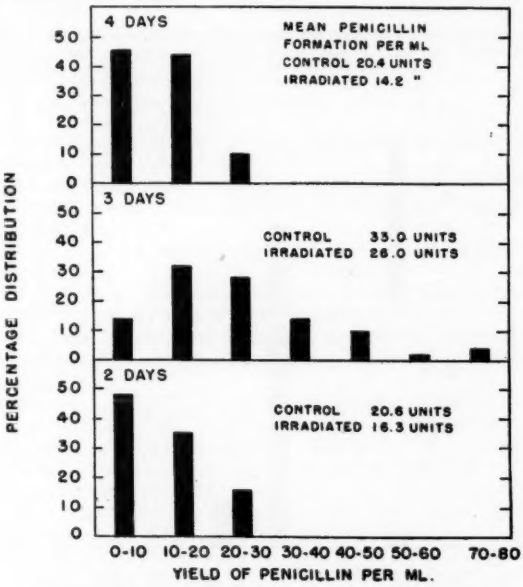

Fig. 3. Per cent distribution against yield of penicillin in Oxford Units based on tests made by Dr. J. W. Foster. Time of tests refers to days of incubation.

The morphological mutations showed wide variety in appearance. Several interesting mutations were found which showed certain deficiencies when grown in Czapek solution agar but which appeared normal on a more complete medium (malt extract agar). One of these is a thiamin-deficient mutation. When grown in Czapek solution agar it forms a thin spreading mycelium, while in malt extract agar it duplicates the normal mycelium. Another mutation appears deficient when grown on a nitrate medium, but when it is grown on a medium with ammonia or amino nitrogen the culture appears normal. A number of other deficiencies have appeared which await analysis.

In a separate study Lockwood, Raper, Moyer and Coghill ('45) investigated 217 irradiated cultures for their ability to produce itaconic acid. It was thought that it would be possible to inhibit some of the enzyme systems which would then leave the organism to ferment a higher percentage of the sugar to itaconic acid. I am quoting from their summary:

> "Nine different types of biochemical and cultural response have been observed from 217 strains of *Aspergillus terreus* derived from irradiated conidia.
>
> "Among the 76 strains which were morphologically unchanged were 59 which appeared to be unaltered biochemically, 13 which produced more itaconic acid than the parent strain, and 4 which produced no itaconic acid.
>
> "Among the 141 strains which were obviously altered morphologically were 42 strains not apparently altered biochemically, 88 which produced little acid, and 11 which did not grow on the test medium. None of these 141 strains produced more itaconic acid than did the parent strain.
>
> "Fifteen strains produced considerable non-acidic unsaturated material.
>
> "Seventeen strains appeared to produce no acid other than itaconic."

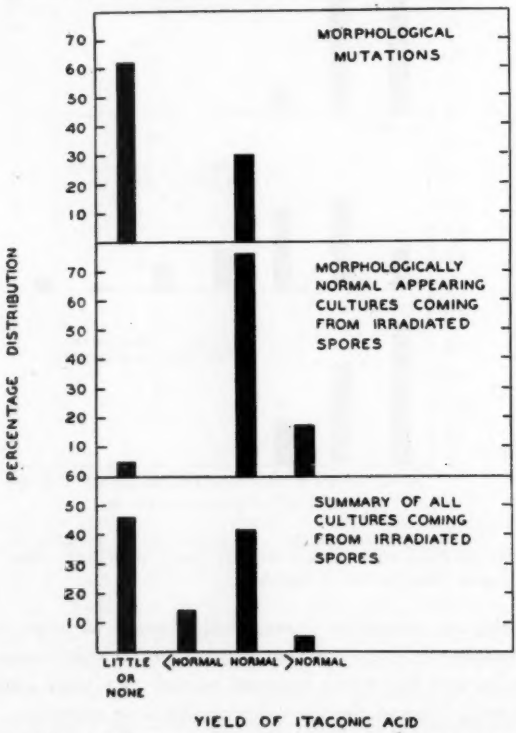

Fig. 4. Distribution of yield of itaconic acid produced by *Aspergillus terreus* mutations based on Lockwood, Raper, Meyer and Coghill ('45).

A block diagram of the percentage distribution of the tested cultures on the basis of yield of itaconic acid is given in fig. 4. The tendency of morphological

mutation to give lower yields of itaconic acid is well demonstrated.

It is not unusual to find in nature strains of *Penicillia* or *Aspergilli*, believed to represent mutations, which have different biochemical activity from the usual standard "accepted" strains; and there is good reason to expect to find in the naturally occurring strains occasionally one with more desirable fermentation properties. This type of mutation might very well have survived by natural selection. Such strains have actually been found with *Aspergillus terreus* (Raper, Coghill and Hollaender, '45). A promising investigation would be the irradiation of these new high-yielding strains and the study of the mutations produced.

If we analyze the data from these three sets of experiments, we can conclude that it is not difficult to interfere with the normal metabolism of an organism. The combination of interferences which would result in an increased production of certain chemicals can not be expected to happen often.

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THE INFLUENCE OF NUCLEIC ACID ON DEHYDROGENASE SYSTEMS A CONTRIBUTION TO THE PROBLEM OF GENE MECHANISM

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INTRODUCTION

The cellular components comprise a vast number of different compounds, salts, proteins, carbohydrates, fats, nucleates, etc., each in a state of metabolic flux, and each interacting with others to provide the energy for specific function, the supply of structural materials, and the disposal of breakdown products. This complicated and highly diverse mechanism of the cell is regulated by a mosaic of enzyme systems which are not independent but interrelated, and which are themselves regulated and affected by extrinsic and intrinsic factors and conditions. Thus, it is possible to affect the level of particular enzymes by nutritive deprivation of the cell, by the application of chemical inhibitors or stimulators, and by cellular mutations whether spontaneous or induced. Any attempt to elucidate these phenomena *in toto* is quite obviously a formidable task. The biochemist at present can study only a few systems at a time, under conditions often considerably removed from the physiological, and, as yet, with tools and concepts alike available only for first approximations. Biological chemistry owes its beginnings to the insight of men like Miescher, Kossel, and others, who believed that problems in tissue function might be at least partially explicable in terms of the chemical properties of isolated components and systems. It is with this hope undimmed, and with due recognition of the limitations inherent in the approach, that an attempt at a study of a chemical basis of gene action may be begun (Greenstein and Chalkley, '45).

Nucleic acid combined with specific proteins appears to yield conjugated compounds often with remarkable biological properties. Among these compounds are the viruses and the components of the chromosomes. Considerable information exists suggestive of a linking of nucleic acids with gene mechanisms, but in the absence of evidence for the isolated gene any attempt to identify the latter as a nucleoprotein must be treated with some reserve. Nevertheless, there is evidence that growth and reduplication are associated with the presence of nucleic acid, and the relation of the latter type of substance with the gene may thus be strongly inferred. Since the genic material exerts a controlling influence over the multitudinous functional processes of the cell, it may be assumed that it accomplishes this not by a remote form of control but by specific kinds of chemical interaction with the cellular components involved in individual reactions, i. e., enzymes (cf. Tatum and Beadle, '45). It is for this reason that the study of the chemical interaction of nucleic acid with various kinds of cellular components should yield sig-

nificant implications (Miescher, '97, Hammarsten and Hammarsten, '28, Greenstein and Jenrette, '41).

In the form of their neutral sodium salts, the nucleic acids in aqueous solution are highly elongated, polymerized molecules (cf. Greenstein, '44). The degree of asymmetry and the extent of polymerization of the desoxyribose nucleate (chromosomal component) are markedly decreased in the presence of proteins and of salts, and the relative amount of this decrease is a function of the nature, the state, and the concentration of the protein and of the salt. The molecular configuration of the nucleates is in particular highly labile toward the proteins.[1]

The question that naturally arises is whether there is some reciprocal effect of the nucleate on the molecular configuration of the proteins with which it interacts. This question is most readily answered by employing proteins with specific and readily measured properties, namely enzymes. Changes in the physical or chemical properties of these substances, due to the interaction with the nucleate, might be expected to be reflected in observable changes in their catalytic capacities. Furthermore, if such changes occurred, they would provide a possible clue to some of the mechanisms which chromosomal components exert in the maintenance and regulation of cellular functions.

PROCEDURE AND RESULTS

Aqueous tissue extracts containing reducing systems possess the capacity of decolorizing methylene blue under anaerobic conditions. We have observed that when sodium yeast nucleate (ribose nucleate) is added to such extracts, the decolorization rate is slightly decreased; when sodium thymus nucleate (desoxyribose nucleate) is added, this rate is very considerably decreased. The extent of this decrease in rate is proportional to the amount of nucleate added. Addition of xanthine results in an increase in decolorization rate (measure of xanthine dehydrogenase activity) which, at high levels of dye concentration, appears to be very nearly the same whether nucleate is present or not. The percentage increase in rate on addition of substrate, however, is greatest in the presence of thymus nucleate. Nearly identical results are obtained with freshly-mixed solutions and with mixtures which are allowed to stand until the viscosity of the thymus nucleate is reduced nearly to that of the extract (enzymatic depolymerization (Greenstein, '44)). The fact that the activity of xanthine dehydrogenase is independent of the presence of nucleate (at high dye concentrations) indicates that there is no effect of the nucleate on the dye. The results of a typical experiment are given in Table I (Greenstein and Chalkley, '45).

When the mixtures described in Table I are treated with smaller and smaller quantities of methylene blue, keeping the total volume constant by addition of water, data indicated by the curves in fig. 1 are obtained.

The exponential character of the curves in fig. 1 led us to replot the data given

[1] This configuration is also labile toward the effects of ultra-violet radiation (Hollaender, Greenstein, and Jenrette, '41), and this phenomenon is suggestive in connection with mutations induced by this agent (cf. Carlson and Hollaender, '44).

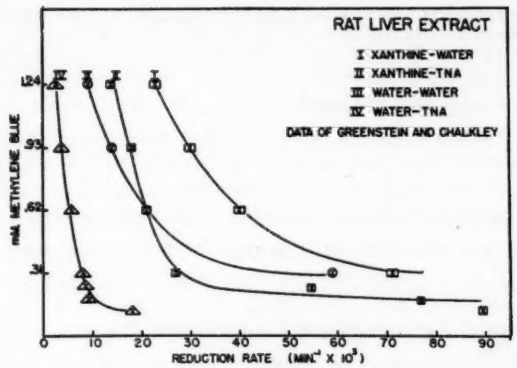

Figure 1. The relation between methylene blue concentration and the rate of decolorization of the dye. Ordinate refers to m M dye per cc. added to mixtures described by curve I consisting of 2 cc. extract + 1 cc. water + 1 cc. xanthine (1.6 m M); by curve II consisting of 2 cc. extract + 1 cc. 0.5 per cent sodium thymonucleate + 1 cc. xanthine (1.6 m M); by curve III consisting of 2 cc. extract + 2 cc. water; and by curve IV consisting of 2 cc. extract + 1 cc. water + 1 cc. 0.5 per cent sodium thymonucleate. Abscissa refers to rate of complete decolorization. Anaerobic conditions throughout. Temperature 24–26° C.

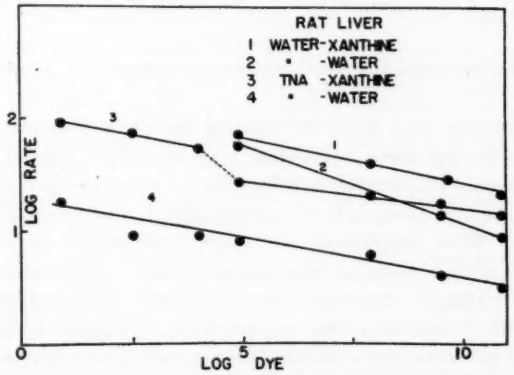

Figure 2. Relation between the logarithms of the methylene blue concentration and of the decolorization rates based upon data given in fig. 1. Composition of mixtures described by curves 1 and 4 are the same as that described by curves I and IV in fig. 1; that of curves 2 and 3 identical with that respectively of curves III and II in fig. 1.

TABLE I

EFFECT OF YEAST AND THYMUS SODIUM NUCLEATES ON THE DECOLORIZATION RATE OF METHYLENE BLUE IN RAT LIVER EXTRACTS*

Mixture†	Decolorization Rate
	Minutes $^{-1}$ x 10^3
1. 2 cc. liver extract + 1 cc. H_2O + 1 cc. H_2O + 1 cc. methylene blue	50
2. 2 cc. liver extract + 1 cc. H_2O + 1 cc. xanthine + 1 cc. methylene blue	83
3. 2 cc. liver extract + 1 cc. yeast nucleate + 1 cc. H_2O + 1 cc. methylene blue	40
4. 2 cc. liver extract + 1 cc. yeast nucleate + 1 cc. xanthine + 1 cc. methylene blue	71
5. 2 cc. liver extract + 1 cc. thymus nucleate + 1 cc. H_2O + 1 cc. methylene blue	7
6. 2 cc. liver extract + 1 cc. thymus nucleate + 1 cc. xanthine + 1 cc. methylene blue	38

* Concentration of methylene blue 1.24 x 10^{-3} M, of the nucleates 1.0%, of xanthine 1.6 x 10^{-3} M; each cc. of the aqueous liver extract equivalent to 300 mgms. of tissue; temperature 24–26° C. Anaerobic conditions throughout by employment of evacuated Thunberg tubes at approximately 18 mm. pressure.

† Xanthine dehydrogenase activity obtained by subtracting, respectively, 2. from 1., 4. from 3., and 6. from 5.

in terms of the logarithms of the respective variables with results described in fig. 2.

It is evident that there is a linear relation for all the mixtures at the higher dye concentrations, but there is a distinct break in curve 3 (fig. 2) at the lower dye concentrations. This break, symbolized by a dotted line since we do not know as yet its shape at this point, indicates an apparent increase in xanthine dehydrogenase activity. With progressively decreasing amounts of the methylene blue this increase in activity becomes progressively greater. The activity of xanthine dehydrogenase, although independent in the presence of desoxyribose nucleate at the higher levels of methylene blue concentration, is affected by the presence of the nucleate at the lower dye concentrations in such a way that it is apparently increased.

This apparent increase in dehydrogenase activity in the presence of the nucleate at low dye concentrations is all the more striking when the activity of this enzyme in the absence of added nucleate is considered. Inspection of curves 1 and 2 in fig. 1 suggests that these curves cross in the vicinity of the break in curve 3. The crossing of curves 1 and 2 means that with progressively lower dye concentrations the activity of xanthine dehydrogenase becomes apparently increasingly more negative. Further investigations have indeed revealed this to be the case. At very

low dye concentrations, the presence of the substrate, xanthine, interferes in some way with the ordinary processes of reduction of the dye by the tissue reducing systems. On the other hand, at these same low dye concentrations, when both substrate and nucleate are present, the rate of reduction of the dye is accelerated. Thus, with decreasing amounts of methylene blue, the divergence in the activity of the dehydrogenase in the presence of the nucleate or in its absence, is in opposite directions, i. e., the activity in the presence of nucleate becomes increasingly greater, the activity in the absence of nucleate becomes increasingly negative. The effect of the nucleate is thus markedly emphasized, and suggests qualitative as well as quantitative effects produced by this substance. No changes in pH are produced by the addition of the neutral sodium nucleates.

No explanation is apparent at the present time for the different directions which the activity of xanthine dehydrogenase takes in the presence and in the absence of desoxyribose nucleate. The results are quite reproducible from one extract to another. Further investigations on other enzyme systems and on other tissues are clearly desirable.[1]

Discussion

At this time any attempt at interpretation of the phenomena above described must of course be speculative. They are, from the biological viewpoint, the initial results of an attempted approach to the problem of the well-known role of the nucleus as co-ordinator of the functional activities of the cell. In this respect, it is perhaps permissible to note that, as far as the writers are aware, the results

[1] Since the above was written, more experiments involving a greater range of dye concentrations have been performed. These have shown that the relative rates of decolorization in the presence of nucleates may actually be accelerated when the dye concentration is sufficiently increased. It will be noted from fig. 2 above that curves 2 and 4 converge as the dye concentration is increased and presumably would cross at a relatively high dye concentration. The reality of such an intersection has now been experimentally established, and thus the existence of an accelerating effect of desoxyribose nucleate at sufficiently high dye concentrations has been proved. A similar study has also been made with respect to ribose nucleate, and it has been found that an analogous reversal of effect occurs, but at a substantially lower concentration of dye than that required for desoxyribose nucleate. Thus, at sufficiently low concentrations of dye the addition of either ribose nucleate or desoxyribose nucleate retards the decolorization rate; at sufficiently high concentrations of dye the addition of either nucleate accelerates the decolorization rate, whereas at intermediate concentrations of the dye the addition of ribose nucleate accelerates, and the addition of desoxyribose nucleate retards, the decolorization rate. The relative ranges of dye concentration where these three separate effects are noted will depend on the tissue used, the degree of dilution of the extract, and on the concentration of the nucleate.

Dilution of the extract may be taken as equivalent to reducing the amount of oxidizable substrate. Changes in dye concentration may be equated to normal hydrogen acceptor levels. Hence it would appear that for any given level of substrate and hydrogen acceptor the level of nucleates within the cell would determine the rate of dehydrogenation (anaerobic stage of oxidative metabolism). Since fluctuations in the release or production of nucleates within the cell may reasonably be assumed to be associated with gene action, we may be here dealing with the normal mechanism for control of general cellular metabolism—at least in so far as the initial stages of oxidative metabolism are concerned. The system or systems here studied are obviously highly complex and appear to exist in a delicately adjusted state of equilibrium. As a further example in addition to the phenomena listed above, the use of phosphate buffers normally considered innocuous, when added to tissue systems in such a way as not to disturb the pre-existing pH, markedly alters the quantitative aspects of the decolorization rates. Specific salt effects are thus also concerned in the kinetic mechanisms. Investigations are now in progress covering this and related aspects of the problem.

constitute the first direct chemical evidence that a typically nuclear constituent, desoxyribose nucleic (thymonucleic) acid can directly affect the enzymatic redox systems of the cell.

The fact that, in the presence of desoxyribose nucleate, the over-all activity of these systems is altered immediately suggests that differences in production or release of such nucleates within the cell could regulate its metabolic activities. It also appears, since the effect of the nucleate is apparently relatively, if not absolutely, reversed for xanthine dehydrogenase (the enzyme typically involved in purine metabolism) at low oxidative levels (i. e., low concentrations of methylene blue), that this regulative effect might extend to the metabolism of the nucleic acid itself. In that case the effect would apparently depend largely upon the concentration of the hydrogen acceptors available, up to and including oxygen.

One is tempted to recall the finding of Chalkley and Voegtlin ('40), that changes in oxygen tension strongly affect the growth and fission of the nucleus in *Amoeba proteus* and also the sulfhydryl cycle observed by Chalkley ('37) within the nucleus of the same organism, which last could conceivably produce a variation of redox equilibria within the nucleus, i. e., in the immediate vicinity of the desoxyribose nucleic acid, and which was shown to be correlated with the growth and fission of the nucleus. Considered in conjunction with the present data, these observations might lead to the suggestion that the oxygen supply together with desoxyribose nucleic acid metabolism might, through affecting the redox systems of the cell, constitute the regulating mechanism of the entire cell metabolism.

Further, the intimate relation of the desoxyribose nucleic acid to chromosome structure might suggest that gene activity expresses itself in no small part by means of this system.

As stated above, this is obviously speculation but at least there can be no doubt that we have at last definite physiologic, i. e., biochemical, action demonstrated to occur between a component limited to the nucleus and certain enzyme systems concerned in cell metabolism.

It is of interest to note that the ribose form of the acid (yeast nucleic acid) shows, if any, much less retardative activity. Thus the cytoplasmic form of the acid is sharply set off in this respect from the purely nuclear form.

The implications of these extremely simple experiments are far-reaching. It is obviously necessary to carry out greatly extended research, however, before any of the foregoing suggestions can have any merit other than that of serving as indications of our mode of approach.

SUMMARY

Aqueous extracts of rat and of mouse liver possess the capacity of reducing solutions of methylene blue. In the presence of desoxyribose nucleate the rate of decolorization of relatively low concentrations of dye is appreciably delayed, and the decrease in rate is proportional to the nucleate concentration.

At relatively high levels of dye concentration the activity of xanthine dehydrogenase in these extracts is the same whether desoxyribose nucleate is present or not. At low levels of the dye there is an apparent increase in the activity of the enzyme when desoxyribose nucleate is present.

Ribose or desoxyribose nucleate may accelerate or retard the reducing systems of the liver, the effect depending on the dye concentration.

The possible implications of these findings are discussed in the light of genic mechanisms.

Acknowledgment is made of the skillful and devoted assistance of Miss Florence Leuthardt.

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GENETIC ASPECTS OF VIRULENCE IN BACTERIA AND VIRUSES[1]

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At Iowa State College we consider that disease is due to the interaction of four major variables: the genetic constitution of the host for disease susceptibility or resistance, the genetic constitution of the pathogen for virulence or avirulence, the dose of the pathogen to which the host is exposed, and a multitude of variables which we ordinarily include in any genetic experiment under the head of environmental effects. For disease to be produced, the genetic constitution of the host must be a mirror image of that of the pathogen in that a genetic constitution for susceptibility has a relatively low survival value against all organisms whether they are virulent or avirulent. A host constitution for medium susceptibility has a fairly high resistance against avirulent organisms, medium resistance against medium virulent organisms, and a small resistance to the highly virulent type. A highly resistant host has high resistance for all organisms except the most virulent to which they now and then succumb.

HOST MATERIAL

The studies herein reviewed were started in 1925 by differentiating a single host strain for mice and for the domestic fowl into forms highly resistant to *Salmonella typhimurium* and *Shigella gallinarum* respectively. From earlier experiments a dose of 5×10^4 organisms per mouse was chosen as the agent by which resistant strains were established from the previously highly susceptible strains. Similarly for poultry a dose of 1.2×10^7 of the fowl typhoid organism *Shigella gallinarum* was chosen. These organisms were inoculated intraperitoneally. Animals which survived in the best condition in each generation were used as the parents for the next generation. The results of the first fourteen successive generations of selection are shown in fig. 1. Intense inbreeding was used for each group to purify the genetic constitution.

The graphs of fig. 1 show that for both hosts the resistance increased rapidly at first, then somewhat more slowly for six or seven generations, the ultimate survival value of each group being 80 to 90 per cent. In the eighth generation for the mice the dosage of organisms was increased to 2×10^5. This increase was accompanied by a 10 per cent reduction in survival. From that point on the resistance increased again, 93–96 per cent resistant animals being reached in the 14th generation. The results show that despite continuous selection and inbreeding for eight generations there was further residual variation within the strain. Chicks of the eleventh generation were not tested. Subsequent tests showed high

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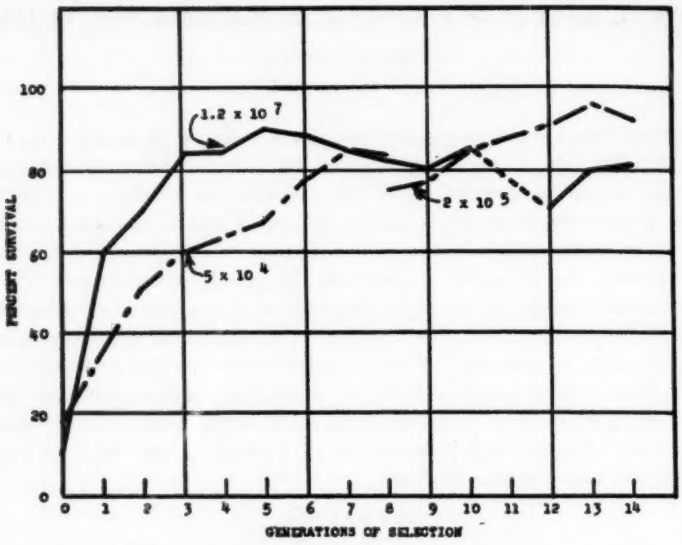

Fig. 1. Survival value of mice (Schott, '31, '32 and Hetzer, '35, '37) and the domestic fowl (Lambert, '31, '36) for successive generations of selection toward the resistant types. Solid line for fowl, dot-and-dash line for mice. Eleventh generation of fowl selection was untested. Eighth generation of mice had dosage changed from 5×10^4 to 2×10^3.

resistance within the chickens. Testing of the parents is not necessary for the resistance in the progeny. The chickens and mice have kept their high genetic resistance despite the fact that the inbreeding has led to the accumulation of genes for smaller size, lower fertility, and some apparent lack of vigor.

Two possible explanations might account for this change in resistance: selecting of small variations due to genes for resistance already in the parental lines or selecting of mutations toward higher resistance, each mutation being partly dependent on the total genetic constitution of the host for disease resistance. Either explanation would lead to the disease resistance observed in these lines. Actually both factors appear important. There is some experimental evidence to indicate that if a sufficiently large population of mice is chosen it is possible to pick out from this population individuals which carry very high genetic resistance to mouse typhoid and actually make the change from a relatively susceptible population to a highly resistant population in a very few generations. This sudden change could favor the view that disease resistance may be accomplished in one step or may be due to a single gene pair. The results of Hetzer show that this is not the case. The circumstances leading to the choice of resistant mice are fortuitous, a result of a combination of several genes for resistance in one animal brought about by chance segregation.

A second question of interest is why a completely resistant strain is not attained. The strains which have been formed would be considered completely resistant if bacteria of low virulence were used to initiate the disease. With highly virulent organisms, some deaths do occur. It seems that no species of animals having a native disease has yet had a completely immune race established through genetic means or any other means for that matter. Highly resistant animals have been produced but with highly virulent organisms of the pathogen it is possible to produce some deaths in all cases.

Since the fourteenth generation the selected strains have been maintained without testing. The resistance of the present generation is as high as it was under testing. Genetic resistance when made homozygous for the strain is a permanent attribute of the strain.

PATHOGEN VARIATION

In November, 1940, our culture of *Shigella gallinarum* was found to have completely lost its virulence when used in a fairly large test. This culture had been highly virulent the previous May. The results demonstrate that a large population of virulent bacteria could be replaced by avirulent bacteria in a period of something like seven months. This fact was of particular interest since this line had previously retained its pathogenicity for several years.

The mechanism of such changes was not entirely unknown to us as our previous studies of such genetic variation of the pathogen had given us a fair understanding of how they came about. The following investigators made large contributions to this problem and to the others herein discussed. The results reported are the joint effort of the following workers in our laboratory: Dr. M. R. Zelle, Miss Janice Stadler, Mr. G. W. Kohler, Mr. John A. Weir, Mr. A. E. Bell, Mr. E. F. Oakberg, and Dr. R. E. Lincoln.

The avirulent culture was subjected to the proper cultural and serological studies to prove that it was *Shigella gallinarum*. The culture was then inoculated into a chicken of a very susceptible strain. Five different isolations were made from this host. Two of these isolations did not progress very far, one being lost in the first passage and the other one shortly thereafter, indications of the low virulence of the *Shigella* culture.

Isolations were made from the heart, liver and spleen respectively. As no small chicks were available, these lines of bacteria were passed through six successive 10-week-old birds of the susceptible strain, each line being kept separate from the others.

The bacteria were kept in the chickens one week, then for three days on culture media at each passage. The inoculating dose was two billion organisms. These passage birds showed no mortality, but the organism was recovered from each bird inoculated. Tests for virulence on the seventh passage organisms were made on 10-day-old chicks. One line, D7, killed 10 out of 10 chicks in less than 10 days. The second line killed 6 of 11 chicks but took 21 days to do it. The third line

killed 8 of 11 chicks but also took 21 days to do it. A transfer culture of the parent avirulent culture from which the above lines originated killed 3 of 11 chicks in 21 days. It is evident that one of these strains, D7, differs from the others in virulence.

Further analysis of line C showed that for an average dose of 5×10^6 it killed only 6 out of 35 resistant chicks and 11 out of 37 susceptible chicks. Line D killed 13 out of 41 resistant chicks and 36 out of 36 susceptible chicks. Line E killed 11 out of 40 resistant chicks and 34 out of 34 susceptible chicks. The percentage comparisons were for the resistant line 17, 32, 27, and for the susceptible chicks 30, 100 and 100, for lines C, D and E respectively. The parent avirulent culture showed 24 per cent mortality in the susceptible host. Two relatively pathogenic lines had been established from a highly avirulent line. The mechanism of this selection is important.

To determine the variability of *Shigella gallinarum* under natural conditions, a survey was made of cultures from chickens diagnosed as clinical fowl typhoid during the summer. Sixteen cultures and 11 sub lines showed marked variability in the end point for agglutination in anti *Shigella gallinarum* and/or anti *Salmonella typhimurium* serum, metabolism of sugars, colony morphology and pathogenicity. The species *Shigella gallinarum* evidently had wide genetic variability.

ANALYSIS OF VIRULENCE CHANGES

Experiments were planned to analyze bacterial variability as it is related to the genetics of virulence. From the avirulent stock culture described above 20 colony isolations were made. As this organism does not clump appreciably, each of these colony isolations probably represents the descendants of a single bacterium. Ten of these avirulent lines were exposed to the environment of our inbred, highly resistant chickens described above. These inbred lines are capable of surviving nearly 1000 times the number of bacteria which will cause death in most flocks. The other ten strains of avirulent bacteria were grown in a strain of chickens marked by susceptibility to fowl typhoid. Two chicks were used at each passage for the resistant host line and one chick for the susceptible host line. The avirulent strains of *Shigella gallinarum* were thus exposed, on the one hand, to the intensely unfavorable environment of the resistant strain of host, and, on the other hand, to the more favorable environment of the highly susceptible host.

Attempts were made to pass each culture successively through 16 different 10-day-old chicks using the technique described above. Despite the fact that twice as many chicks were available for recovering the organism at each resistance passage, 24 passages were lost in the transfers through the resistant host compared to 10 for the susceptible series. Life for the typhoid bacteria in the resistant host was tough. The avirulent strain has great difficulty in establishing itself even to making a mild disease in the resistant host strain. This fact suggests that the resistant host would be a potent selecting force tending to pick out the progeny

of any variants characterized by increased virulence.

Small tests were made throughout the passage experiments to determine the constancy with which the organisms recovered retained their virulence. A larger test was made at the end of the experiment to establish more exactly the virulence of each line. The rather scattered and low amount of data taken during the passage of the 20 lines of bacteria through their respective hosts show that each strain retained its low virulence for a varying number of passages. Changes when they did occur came suddenly during a single passage and resulted in a substantial gain in virulence, the total amount of change differing for different strains. When a change in virulence did occur, the subsequent tests showed a retention of the new virulence. These results favor mutation and subsequent replacing of the avirulent type by the virulent mutant.

Tests of the 20 lines at the end of the sixteenth passage give further support of this conclusion. Two of the lines, I and R, had not changed in virulence as the result of growing in their natural host for half a year (fig. 2). One line was carried in the resistant host. The other line was passed through the susceptible host. If virulence is due to chance mutation, the expectation would be essentially equal numbers of mutations in each group. The observations bear out this hypothesis. Two lines of medium virulence have been established from the resistant host against three lines for the susceptible host. Seven highly virulent lines came from resistant host passage and six from susceptible host passage.

The over-all picture for the 10 lines passed through the resistant hosts was as follows: A dose of 100,000 organisms inoculated into 74 resistant chickens led to 20 per cent death; inoculated into 203 susceptible chickens led to 70 per cent death. With 100,000,000 organisms as the dose, 70 resistant chickens had 22 per cent death, 124 susceptible chickens had 88 per cent death. For the lines derived by passage through the susceptible host the 29 resistant chickens with 100,000 dosage had 7 per cent death and the 186 susceptible chickens had 84 per cent death. For the 100,000,000 dosage 54 resistant chickens had 22 per cent death, and 98 susceptible chickens had 86 per cent death. These data show that passage through either host is equally favorable to establishing of virulence. The degree of increase in virulence may be judged by the fact that the original avirulent culture inoculated in 100,000,000 organisms showed no death on the resistant host and only 34 per cent on the susceptible host.

The chicks of either strain are highly efficient selective agents favoring any variants toward virulence and encouraging them to multiply at the expense of the avirulent type. The population within the host becomes rapidly purified towards the virulent type. The genetic constitution of the domestic fowl, the natural host to this disease, is sufficient to create the necessary conditions for this selection process. The culture media, on the other hand, appears to favor those organisms whose genetic constitution is for a saprophytic type of growth.

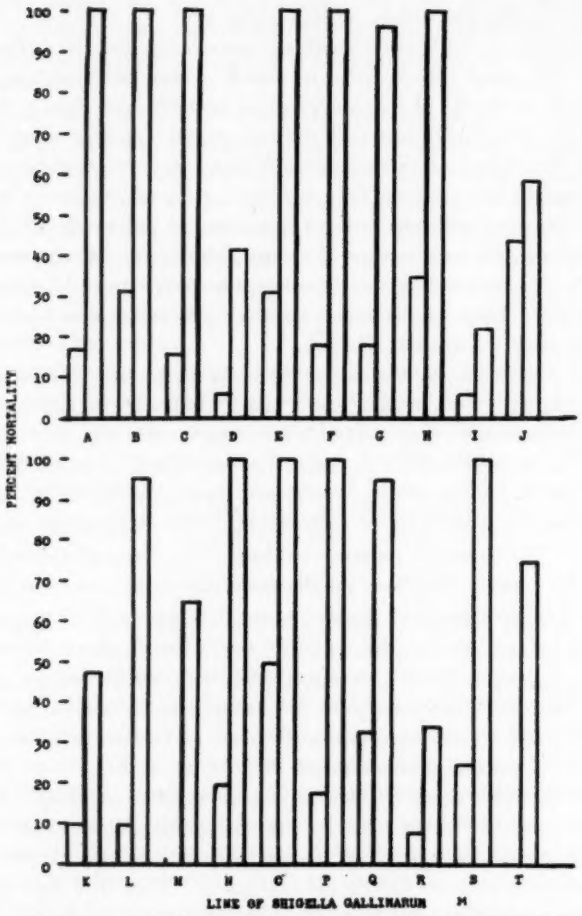

Fig. 2. Virulence of different lines of *Shigella gallinarum*, originating from the same avirulent line, after 16 passages through resistant chicks A to J and susceptible chicks K to T. Left, ordinate tests on resistant chicks; right, ordinate tests on susceptible chicks.

GENETICS OF VIRULENCE IN MOUSE TYPHOID

Experiments of a similar nature were carried on earlier in this laboratory utilizing *Salmonella typhimurium*, the agent of mouse typhoid. A single laboratory line of *Salmonella typhimurium* was available. This line had retained constant virulence on culture media for more than ten years. Six different experiments were performed, each varying somewhat, but all directed toward detecting and tracing virulence changes in this line. Six different strains of mice, differing in their resistance to mouse typhoid, were available.

In some experiments the bacteria selected for use were the direct descendants of a single organism picked out by the micropipette. In others, the organisms were the result of five successive platings and single colony isolations. The initial bacterial line chosen had essentially the same virulence as the parent culture. The parent culture was different from that described for the domestic fowl in that it was originally a culture of medium virulence.

The culture was divided into two parts: one part exposed to the effects of the environment of the resistant host strains of mice, the other part to the environment of susceptible host strains of mice. The longest experiment performed involved 36 successive passages of bacterial line from one mouse to another and covered a period of two years. The outcome of these experiments brought out several facts important to our interpretation of the physical basis for virulence of a disease-producing organism.

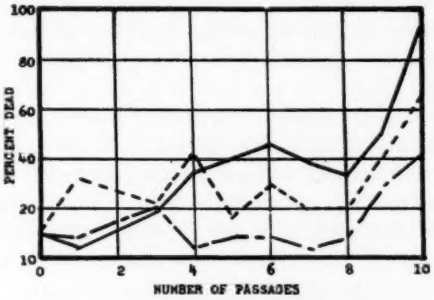

Fig. 3. Changes in virulence observed in the passage of a medium-virulent line through susceptible mice, through resistant mice, and as kept on culture media. Solid line is for the passage through resistant mice; dotted line, for passage through susceptible mice; and dash-dot line, for the culture on culture media. Tests made on resistant mice.

In general, the stability of our original pathogenic line was demonstrated. Increased virulence, when it was observed, occurred only in a low proportion of cases following passage of recently isolated single-celled cultures. Changes in virulence, when they did occur, were sudden. The increased virulence observed was then subsequently maintained at the new high level. There was no suggestion of a gradual accumulative effect of the environment on these increases in virulence. Lines of the pathogen more virulent than the parent culture were obtained in passages through both resistant and susceptible hosts. If virulence increased during passage, the increase was abrupt. Continued growth in the particular host environment resulted in no further increase in virulence. Figure 3 shows one of these experiments.

As bacterial lines were isolated they were sometimes marked by morphological characters along with virulence differences. This association suggests that the phenotypic character of the bacterial colony may be an expression of its virulence.

The correlation is high but not complete. Most but not all of the lines isolated and showing increased virulence went toward the smoother types.

By making use of two bacterial lines differentiated in their colony phenotypes, as well as by their virulence, it was possible to show a very intense selection for the more virulent line of bacteria. Bacteria from the two lines were mixed in definite proportions and then inoculated into mice. At intervals after inoculation bacteria were recovered from the mice, and the proportion of the two types determined. The results showed that the virulent forms quickly became dominant and often were the only type present in the population.

A further confirmation of this fact comes in experiments in which virulent bacteria are inoculated as against those in which avirulent bacteria are used. The bacteria are recovered easily from the host inoculated with virulent culture but with difficulty when inoculated with avirulent organisms.

By growing a large progeny of a single-celled isolation on agar media, it was possible to isolate five different phenotypes appearing as variants of the original culture. Four of these variants showed colonies of greater roughness than the parent. In one the colony was smoother than the parent. This line was also unstable in its phenotype, occasionally producing rough variants. Tests of these phenotypic variants showed that some differed from the parent culture in virulence. The observed facts thus demonstrate the occurrence of phenotypic variations in the progeny of single cells. The amount of this variation is sufficient for the changes in virulence observed in our experiments. Changes in virulence in artificial media are of the same type as those observed in the host. They appear suddenly with sharp differences between the phenotypes.

No relation was observed between the virulence, growth rates, or fermentation reactions of the lines.

In essence the results show changes in virulence to be analogous to mutations in higher forms. The rate of mutation for a given type is small. The changes are sporadic in their appearance and when they do occur are permanent and true-breeding. The variation can go in each direction—toward higher virulence or toward avirulence. The environment of the host or culture medium acts as a selective agent for the genetic type which fits the environment. The environment is not the cause of the variation.

MUTATIONS IN *PHYTOMONAS STEWARTII* AND THEIR RELATION TO VIRULENCE

The problem of virulence and its dependence upon the inherited bacterial constitution may be studied by searching for phenotypical variants in an original pure stock. These variants may occur naturally, under irradiation or in other ways. Two different lines of a corn-wilt organism *Phytomonas stewartii* have been examined for mutations which occurred naturally and after irradiation with X-rays. The first of these lines is a dark yellow rough type with a medium-sized colony. The second type is a large colony with diffuse center. The virulence of these parent lines may be judged by comparing the green weight of plants inocu-

lated with them as contrasted with that of the normal uninoculated plant. Twelve mutants of the dark yellow rough type parent were compared in virulence with the parent type. These mutants were of several kinds. The colonies might be pale yellow, white, roughs of several grades, extreme smooths, mucoid or dry, large or very small. Some of these mutant types, photographed at the same scale and age, are shown in fig. 4.

One parent type had a virulence index of 31, or it was rather low in virulence. Of its 12 progeny mutants 3 were below the parent and 9 were above the parent in virulence. The virulence indexes ranged up to 70. The average was 45. Virulence variations sometimes accompanied the morphological variations and were apparently an expression of the sudden change in type.

Eight variations from the other parent type were selected on the basis of like characters. This parent type had a virulence index of 75. Seven of the 9 mutants tested showed virulence indexes below that of the parent, ranging to as low as 46. Two had indexes above the parent, 81 and 78. The average virulence was 62. The abrupt phenotypic changes in bacterial type observed in this line likewise may affect virulence.

Certain apparent correlations are evident in this comparison. The mutants tend to remain fairly close to the parental type in their virulence. The variation which is observed seems to be directional. When the original parent stock is of rather low virulence, a mutant is most frequently of a somewhat more virulent type. When the parent is of virulent type, then the mutants tend to show less virulence than the parent.

VIRUS MUTATIONS AND PATHOGENICITY

Several investigators working with viruses have noted changes in strain type and in pathogenicity. The analysis of these changes has come particularly in the study of the tobacco mosaic viruses where McKinney noted that suddenly appearing yellow types might be due to mutation from the original form. Jensen isolated over fifty of these variant types occurring normally in ordinary tobacco mosaic. During the course of our own studies on tobacco mosaic large numbers of different variant types have been obtained. These types may be grouped into three major categories: those similar to ordinary tobacco mosaic, those similar to aucuba mosaic, and those producing yellow-mottling rather than the green type. Besides these differences, there are quantitative variations in invasive capacity which seem to be characteristic of the individual variants.

Attempts have been made to determine the inactivation rates of some of these mutants under similar X-ray treatments. The results of these studies indicate that within the limits of accuracy of the X-ray determinations the inactivation rates of the different mutations are the same as those for the parent type. As the different variant types originated from the same parent type, it follows that in so far as this property is concerned, it has been preserved in the variants while they have varied in other directions, i. e., invasive power or phenotypic expression of the host plant. If we take the view that inactivation rates under the same

conditions measure the reproductive size of the virus, it would follow that the size of the virus particle had not changed while the mutation was taking place. The mutation could not be accounted for as splitting of the original particle or as a polymerization to a larger size. Thus the virus may mutate in one characteristic while its other characteristics remain the same as the parent.

Holmes has made a most important study along these lines. He has studied the mutations derived from a masked strain and from a distorting strain of tobacco virus. Thirty-one yellow variants from the distorting strain and 84 variants from the masked strain were observed. Twenty-three out of the 31 variants from the distorting retained fully the systematic invasive power of the parent type. But three of the variant strains produced only local lesions. The mutants from the masked strain, on the other hand, produced no fully invasive types in the 84 which were examined. The changes to the yellow mosaic type in the variants are independent of the invasive characteristic and may represent unit differences in the structure of the viruses similar to such differences in particular genes. These results agree with ours on *Phytomonas* in showing a persistence of virulence type even with marked changes in other characteristics.

These facts gather added significance if the hypothesis that each of these viruses is a unit or molecule is accepted. It would mean that an individual virus may have several side chains or like structures which are capable of affecting the host phenotype in different ways. It will be remembered that tobacco mosaic particles take the form of a long rod composed of smaller units seemingly repeated throughout its length. A single particle could then get its different properties either by different structures of the whole or by having each unit so differentiated as to be responsible for a given reaction.

Each of these different variants of a parent type evidently retains the common characteristics of the capacity for self-reproduction. Added to this basic character, permanent structural alterations may lead to yellow vs. green mottling or localized necrotic lesions vs. the spreading invasive type, etc.

The reproductive capacity is subject to modification by radiant energy and other means. Loss in infectivity may take place under the action of X-rays or ultraviolet light without changes in other properties sufficient to be detected by serological or other means. The presence of these properties is not an indication of the capacity of the virus to reproduce.

Serological techniques of precipitation, complement fixation, or virus neutralization may be used to distinguish different viruses from each other as shown by Chester and others. Strains within a given virus are not readily detected by these means even though these strains show markedly different phenotypic characters in the host plant. These facts indicate that various changes may occur and not be reflected by serological reaction. This may be expected if the particular alteration necessary to produce the new type is not of an antigenic nature. On the other hand, the fact that the larger differences of tobacco mosaic and aucuba mosaic and the enation mosaic may sometimes be detected by serological means indicates that at least some of the changes within these forms are antigenic in nature. One

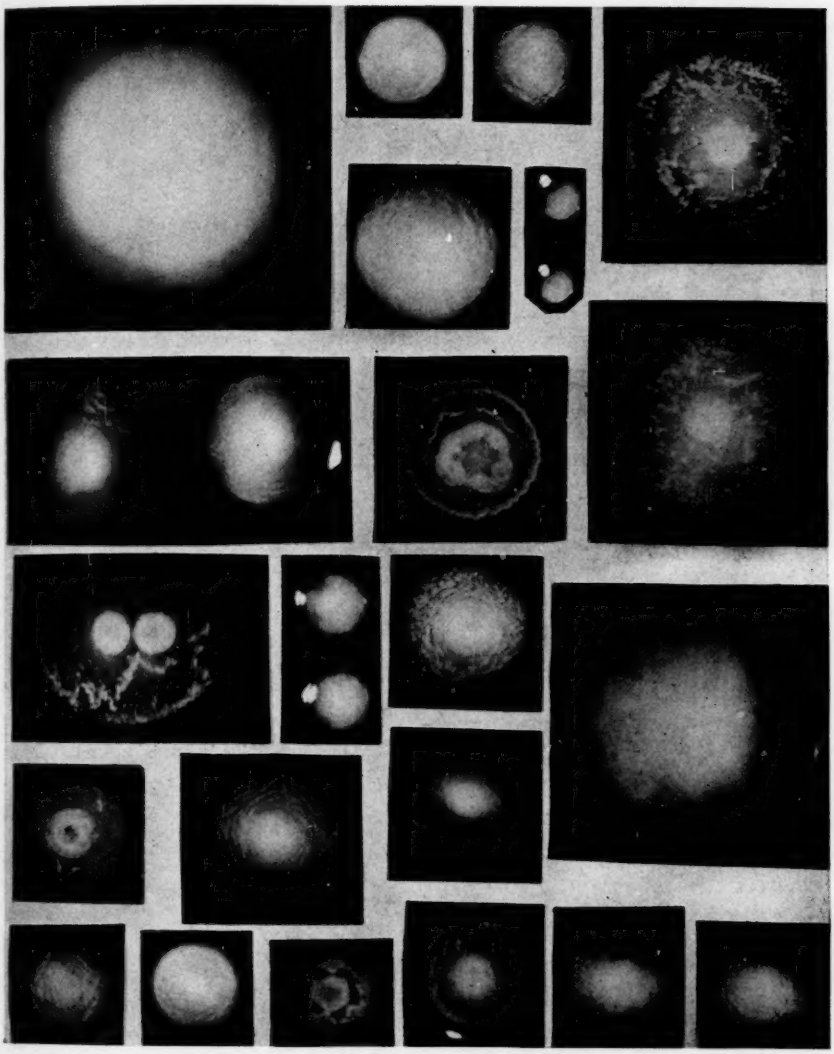

Fig. 4. Mutant types from two lines of *Phytomonas stewartii* differing in virulence.

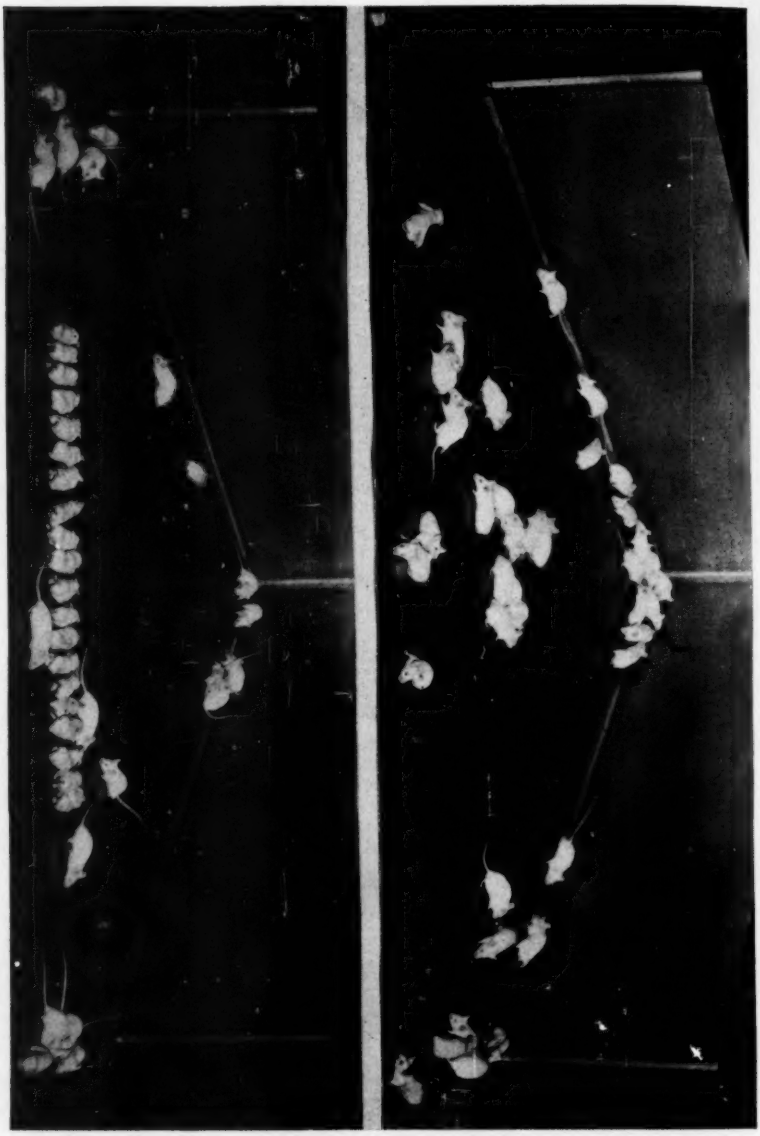

Fig. 5. Photograph showing 20 mice each of the S and Ba strains inoculated with like dose of the same typhoid culture. All the S mice survived; all the Ba mice died.

might assume that such changes involve some type of polysaccharid rearrangement. These facts have a direct bearing on the gene and the detection of its mutants. They suggest that extreme variants of the gene might be detected by a change in its serological structure. Less extreme variants appear much less likely of detection. Under such circumstances the products of the gene's action rather than of the gene's own structural alterations appear more likely of detection.

ORIGIN OF VARIANTS

Variants which occur in cultures *Shigella gallinarum*, *Salmonella typhimurium* and *Phytomonas stewartii* appear to fall in two distinct categories: those in which the variant is stable from the time of its appearance and those in which the variant breaks up into two types, one of which is stable, the other of which continues to break up into the two types in successive generations. These two categories appear to form a discontinuous series and there is reason to believe that the mechanism behind the changes may be distinct.

The time of bacterial mutation in *Salmonella typhimurium* has been traced under microscopic observation. A smooth mutant type was chosen. This type is called unstable as its colonies are composed of bacteria which, on one hand, give smooth colonies and, on the other, give rough colonies. Bacteria from the rough colonies give nothing but rough. The smooth type will repeat, giving both smooth and rough colony types. The case is therefore comparable to similar unstable mutant types in *Drosophila*.

A single organism of the smooth type was picked up in a micropipette, and placed on a thin agar film under the 4-mm. power of the microscope. This cell divided and the daughter cells were separated from each other with the micropipettes. The cells divided again and were again separated. In this manner it was possible to separate and mark the individual cells of six successive divisions. The cells were then allowed to grow into micro-colonies. Each micro-colony was separately picked off, the cells separated by shaking in liquid media and then seeded on agar plates to identify their types. If the colonies were of three types—smooth, mixture of smooth and rough, and rough—the original cell was the smooth unstable type. If the colonies were all rough, the original cell was a mutant to the rough type.

In two such pedigrees a single mutant rough colony was observed, a rate of change of 1 in 134 cells. The sister cell to the rough mutant was smooth. The fact traces the mutations to the events occurring in the division of a single cell into two cells. It shows that the change is not due to any over-all environmental effect for even sister cells do not share the same effects.

The rate of mutation may be checked by a statistical analysis of the relation between the smooth type and rough type found in a single-celled culture after a certain lapse of time where the generation time is known. This statistical analysis will not, of course, substitute for the visual analysis above in showing that the variants occur as one daughter cell of a pair at a single division. Ten separate single-cell isolations were analyzed for their rates of mutation.

The results of this comparison give an average estimate of the mutation rate of 0.0053, or 1 mutation in 187 cells. This agrees rather well with the estimate from the pedigree cultures of 1 in 134 cells.

Observation of a variant appearing as the result of a single cell division has thus been made. The rate of occurrence of these variants is, however, much higher than that observed in the stable variants. An examination of temperature effects on the rate of occurrence of these variants indicates that the mechanism involved for the unstable type as contrasted with the stable may be quite different. In fact, it may be similar to that observed in crossing over or in variegation in *Drosophila*. The results make it highly probable that there are at least two methods by which the variants in bacteria occur.

HOST CONSTITUTION AS RELATED TO BACTERIAL CONSTITUTION IN DISEASE

Three lines of mouse typhoid have been preserved as the result of the above experiments. One is highly virulent; the second is of medium virulence; and the third is nearly avirulent in our customary dosage of 200,000 organisms. The host in which these bacteria have worked have likewise been segregated into strains, the survival value of each particular strain being shown in Table I. Photographs of the outcome of a recent experiment involving 20 mice each of the S and Ba strains strikingly illustrate these differences.

TABLE I

COMPARATIVE RESISTANCE OF Ba, L, E, Z, RI, AND S STRAINS OF MICE TO *SALMONELLA TYPHIMURIUM*, STRAIN 11c, INOCULATED WITH 200,000 ORGANISMS, DATA 1938 TO 1942

Strain	Lived	Total	Survival %
Ba	35	452	8
L	63	470	13
E	292	555	53
Z	733	1262	58
RI	372	496	75
S	988	1150	86

The resistance differences in these strains are genetic. They have been segregated into them and made relatively pure by various means: inbreeding, selection and inbreeding, etc. The strains have held their respective resistance levels for eight or more generations when tested with the same line of mouse typhoid bacteria.

THE CHARACTER BASIS OF VIRULENCE

From the mutations in virulence described above, three lines of the pathogen were preserved. One line is highly virulent, a second has medium virulence, and the third is nearly avirulent in our customary dosage of 200,000 organisms. Hypothetically, virulence in a pathogen could be due to (a) an accentuated capacity for growth to a point where sheer numbers overwhelm the host, or (b) the organism having the capacity to produce a toxin to the host. Some insight into this question was obtained by comparing the lethal action of living and heat-killed cultures (56° C.) of our three bacterial lines. Under these conditions the six strains of mice retained a similar order of resistance to the heat-killed organisms that they had to the live organisms. This indicates that resistance to toxic substances produced by the pathogen is certainly one of the characters involved in genetic resistance or susceptibility. Post mortem examinations, showing that the dead organisms may produce sterile lesions comparable to those observed in the same strains inoculated with the same line of living pathogens, give further support to this view.

However, capacity to grow in the host is also a factor. This is shown by the comparison of the mortalities for the three bacterial lines, when alive or dead. Two of the bacterial lines, differing markedly in live-organism virulence to all mouse strains, showed little difference when compared on a heat-killed basis. Growth rates of the three bacterial lines are equal so that rate of growth of the organism is presumably not a factor. Rather it is the capacity of the organism to grow in the host to numbers which will be lethal. The most lethal bacterial line can grow in mice to the toxic limit. The second pathogen is stopped by the host's resistance before this point is reached. Two genetic characters are thus of demonstrated significance to pathogenic bacteria; the capacity to elaborate toxic products and to multiply rapidly in the host.

CHARACTER BASIS FOR ACTIVE AND PASSIVE IMMUNITY

Mice from the six strains were immunized by inducing clinical typhoid by the live-organism route. The three bacterial lines were used. These mice were subsequently inoculated with a large dose of a single bacterial line. The follow-up dose of live organisms necessary to get a fair death rate is about the same as that necessary to get similar death rates in immunized mice treated with killed organisms. This fact indicates that although antibodies present in the immunized host are able to check the growth of moderate numbers of pathogens, the ability to withstand the toxins produced by the bacterial cells is not greatly altered by the previous immunizations.

But individual host-strain differences appeared on immunization. Using a factorial design some 2700 mice were immunized with killed cultures of three different lines of our bacteria. The mice were equally divided among three different strains, one highly susceptible to typhoid, another of intermediate susceptibility, and a third of great resistance. These mice were immunized once, twice,

or three times. The number of bacteria given at a dose was 1.25×10^7, 1.25×10^6 or 1.25×10^5. The whole design containing 27 treatments was completely balanced, equal numbers of mice being present at each treatment. After immunization and a lapse of 21 days the mice were injected with a rather massive dose of one of the bacterial strains, 5×10^7 organisms of 11 C. The results of these experiments bring out two interesting facts: 1. The three genetically different strains of mice show the same relative resistance to the typhoid organisms after immunization that they had prior to immunization. The dose necessary to bring about death, however, was about a hundred times greater after immunization than before. 2. The bacterial line of low genetic virulence was poor in immunizing capacity. The two virulent lines were both reasonably good immunizers. The results are shown in the succeeding table:

TABLE II

RELATIVE RATES OF SURVIVAL OF IMMUNIZED MICE AFTER INOCULATION WITH 5×10^7 ORGANISMS OF THE VIRULENT CULTURE

Immunizing Organism	Strain of mice			
	Ba	Z	RI	Mean
9 D	8.1	11.0	10.5	9.9
11 C	2.7	37.5	66.3	35.5
DSC1	5.8	30.9	60.5	32.4
Mean	5.6	26.5	45.8	25.9

CHARACTER BASIS FOR INHERITED HOST RESISTANCE

Our studies have shown that genetic resistance to mouse typhoid pathogen can be split up and the host differences segregated into pure breeding strains each characterized by a particular resistance. What inherited characters in the host are responsible for this resistance?

THE BLOOD CELLS

Our first study dealt with the characteristics of the blood cells. These studies showed that numbers of leucocytes were high in the highly resistant lines and low in the susceptible lines, the intermediate lines falling in between these extremes. The correlation between leucocyte numbers and resistance was high. The type of leucocyte did not seem to be of much importance; rather it was the total number. This suggests that the body can call out the type of leucocytes it needs to meet particular environmental circumstances. The numbers of leucocytes are an inherited character of the strains.

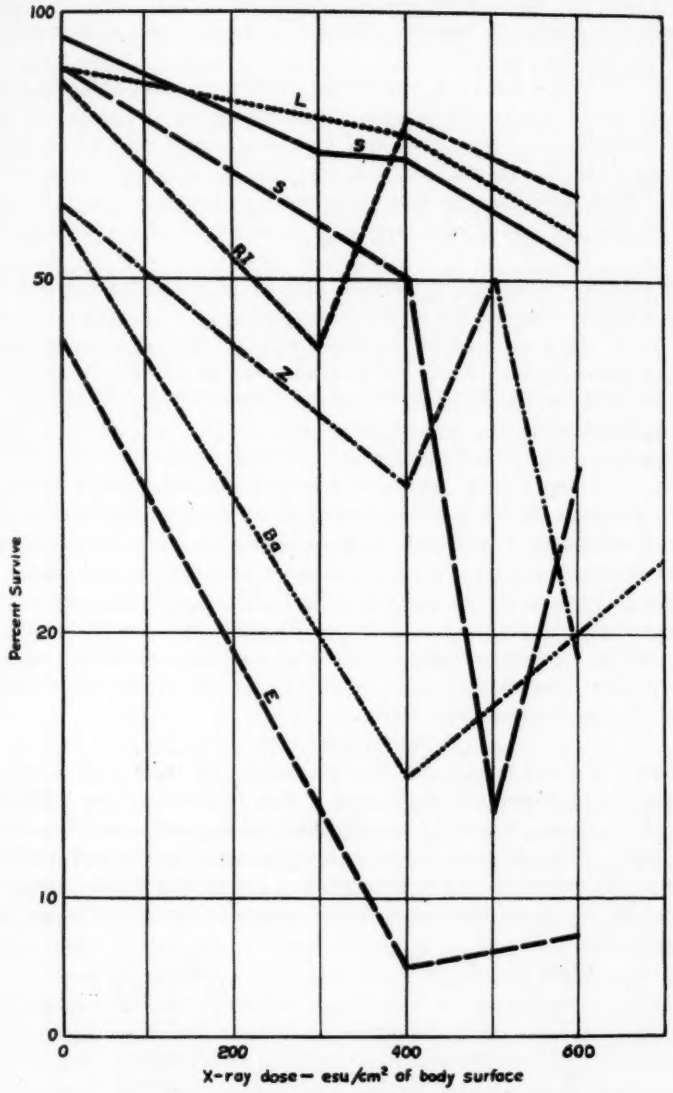

Fig. 6. X-ray treatment of different strains of mice as a means of reducing their resistance to mouse typhoid.

The erythrocyte numbers of the blood are also inherited and fixed for the different lines but the numbers fixed in the different strains have no correlation with leucocyte number or resistance. This is as we might expect if the erythrocytes play no part in the immunity.

X-ray exposure is known to modify the numbers of blood cells. Suitable use of X-rays should thus furnish further information on the part these cells play in the genetic resistance. Some 1256 mice have been treated with X-rays for these experiments; the dose ranged from 0 to 700 e.s.u. per square centimeter of body surface. With more than 700 e.s.u. the mice are so adversely affected as to show severe damage; above 1200 e.s.u. many mice die as a result of the X-ray treatment alone.

The mice were irradiated at about 52 days of age, then allowed a period of 8 days in which to recover from any immediate damage. At about 60 days of age, the S, RI, Z, and E strains were inoculated with 200,000 organisms of medium-virulent typhoid culture. The L and Ba strains were so susceptible that they were inoculated with but 100 organisms of the same culture. The results of this treatment are shown in fig. 6.

All seven mouse strains show a pronounced effect of the previous X-ray treatment on the capacity of the animal to survive inoculated mouse typhoid. The data are plotted as the log of the percentage of surviving mice against the X-ray dosage. Irregularities in individual observations occur but the over-all result is a uniform decline in survival as the X-ray dosage is increased. Between strains there is again a variation in the slope of this decline. This is to be expected on purely random grounds. Some measure of its possible significance can be had by comparing the two S strains as these two curves are really tests for but one strain.

It is evident from the plot that the effect of the X-rays on survival takes the form of the simple exponential equation:

$$\text{Survival} = ae^{bd}$$

Where a is a constant, e, the base of the natural logarithms, b, the term measuring the effectiveness of the X-rays, d, dose, measured in e.s.u. The effectiveness of the X-rays, b, for the two like tests on the same strain, S, is —.0009 and —.0023. The variations of our experiments are evidently such that a difference of this magnitude can be interpreted as due to uncontrolled causes. The constants for the X-ray effectiveness of all strains or the general slopes of the survival line are:

Strain	*Slope (b)*
S	—0.0009
S	—0.0023
RI	—0.0004
Z	—0.0015
E	—0.0028
L	—0.0007
Ba	—0.0014

It is evident that the slope constants are all within the same range. In fact, tests for significance of the differences show that the error within each strain .036 with 17 degrees of freedom is larger than the differences between regressions .027 with 6 degrees of freedom. We may therefore conclude that X-rays affect all strains in a similar manner.

This is important confirmatory evidence that the leucocytes are significant to the physical basis of genetic resistance to mouse typhoid. It is well known that X-rays destroy leucocytes. If the absorption of one unit of ray energy is sufficient to cause the destruction of a leucocyte or its primodial cell, then we would expect the leucocytes to decline according to the form, leucocytes $= ae^{bd}$, as the X-ray exposure, d, is increased. There is a linear relation between survival to typhoid and numbers of leucocytes. We should therefore expect that this decline would give comparable declines in the survival of the different strains to typhoid as the X-ray dosages increase. The data fit this view.

ORGAN AND CELLULAR DIFFERENTIATION IN DISEASE RESISTANCE

Clinical and cytological observations of mice which succumb to inoculations of 200,000 bacteria have shown that mice of the susceptible strain develop extensive lesions in the spleen and moderate ones in the liver. Mice of the resistant strain show no necrosis of the spleen and extensive destruction of the liver tissues.

Cytological studies of liver and spleen in inoculated animals show that with the onset of morbidity glycogen practically disappears from the livers of mice with low and intermediate resistance while glycogen storage is normal in the most resistant mice. With progress of the disease, susceptible mice show extreme fatty degeneration of the liver while the resistant strains show degeneration only as associated with the lesions. These observations indicate that the tissues of the resistant host are able to carry on their normal function even in the presence of the relatively large amounts of toxin which must be present to produce the severe hepatic necrosis which is characteristic of resistant strains.

Bacteria are visible in the liver and spleen about four days after inoculation. In the susceptible strains, bacteria always appear and usually continue to multiply until they kill the host. In mice of intermediate resistance the bacteria are present in the liver and spleen but in about half of the mice they disappear by the 8th to 11th day. The resistant mice, on the other hand, appear to destroy the bacteria rapidly for bacteria are never visible in the liver and spleen of most animals. When bacteria are present, they are usually found in definite lesions.

The spleens of the different strains appear to differ in the white pulp. Resistant strain shows more of that part of the organ than the susceptible strain. Such a difference should be directly associated with the number of macrophages per unit area of the spleen for these cells evidently arise from the lymphocytes of the white pulp both normally and during the progress of the disease.

The genetic capacity to resist or be susceptible to mouse typhoid evidently depends upon several different types of organ and cellular reaction. The particular

types are fixed within the strain by their genetic constitutions.

It might be thought that the humerol elements in the blood might also vary from strain to strain and play a part in disease resistance. Studies have been made on the agglutinative power and also on the bactericidal power of the serum of different strains.

AGGLUTININS AND DISEASE RESISTANCE

It is conceivable that the genetic selection and controlled breeding of the resistant lines could have led to fixation of natural agglutinins to *Salmonella* within these lines. If this were true, their immunological differences could be accounted for by such differences. Tests for natural agglutinins have been carried out on more than 100 mice of each strain but none have been found. Natural agglutinins seem to play no part in the genetic resistance observed in our strains of mice.

BACTERICIDAL POWER OF THE SERA

The natural bactericidal power of the blood could also have played a part in the genetic resistance. Tests for it in more than 60 mice of each strain have also shown it lacking.

GENERAL VIGOR AND DISEASE RESISTANCE

Since the days of Hippocrates it has been thought that some over-all element of disease resistance as general constitutional well-being played a definite part in resistance or susceptibility to many different diseases. While we have shown that such a general over-all condition does not seem to play any part in the resistance to unrelated diseases, it has seemed worth-while to examine the question for the typhoid organism.

Duration of life appears to be a good measure of vigor. A study was made of the duration of life of our six strains of mice. These studies show great differences in the length of life. Some strains are short-lived, others long-lived. Search for infectious causes of death have failed to reveal any of the common disease agents. At 60 days of age one could not pick out the long-lived strains from the short-lived strains by their appearance. In fact, in ordinary life, where internecine strife is a contributing cause of death between the males, one of the short-lived strains is a constant winner. The ability to survive is a clear-cut inherited difference.

This character has a high correlation with resistance to typhoid. The long-lived strains have high resistance; the short-lived are susceptible. Something in the genetic make-up of these long-lived strains favors resistance to typhoid even though previous contact with the organism has been wanting.

DISCUSSION AND SUMMARY

Before attempting an explanation of these results it may be wise to summarize them. For the pathogen a given organism may gain or lose virulence with equal

suddenness. The gain or loss of virulence may extend to very large populations if sufficient time elapses and the selection pressures are great enough. The changes are entirely comparable to mutations in the phenotype of higher forms. Bacteria or viruses may mutate to a multitude of various different types which later will breed true to the new type. These facts point to a relatively large number of genes, with capacities for variation within the pathogen. Some of these genes may mutate independently of virulence. The mutation of others may change the virulence type of the organism. This, too, would be expected from evidence on *Drosophila;* visible mutations may sometimes affect sterility whereas other mutations have no effect on sterility. There seem to be two rather distinct types of these variant changes. One of these has progeny showing only the variant type; the type is stable to the mutant type in the sense that most mutations in higher forms remain stable. Another type has progeny which show both the variant type and a new type. Progeny of the new type remain stable to the new type. Progeny of the variant continue to break up in successive generations to the variant type and to the stable type. These two types of variants, stable and unstable, occur with fair frequency. Temperature effects on the rate of change of the two types suggest that the mechanism behind the mutation processes may be different for each.

Mutations in tobacco mosaic virus show a pattern similar to that of bacteria. The mutations may or may not affect virulence. The different stable mutants observed from a given parent strain are frequently difficult or impossible to separate serologically from the parent strain. On the other hand, widely different virus strains may show three or four antigenic types. These facts suggest that tobacco mosaic virus, although possibly a single molecule, may have multiple antigenic properties, despite the fact that a mutant may not always be distinguished from its parent type in this respect.

These facts have important bearings on gene structure. They would seem to show that if a gene is likewise of molecular type it could have side chains, one responsible for one set of phenotypes and another for another set, the different sets seemingly affecting quite different processes. Such a model of the gene is not the one commonly drawn from the evidence on other forms. The tobacco mosaic units have a structure which suggests another possibility. The tobacco mosaic unit is seemingly built up of sub units, i. e. 2.2 mμ x 2 mμ x 2 mμ or perhaps more probably 37 mμ x 15 mμ x 15 mμ; the larger molecules being the multiples of this type, i. e. 300 mμ in length. The variant types could each be associated with a different sub unit, the whole being more like a chromosome in structure as Bawden has also suggested. The fact that the nucleic acid in each is of different type does not invalidate this parallelism but rather emphasizes the significance of the two models.

For an examination of how this variant behavior of the pathogen affects the host, we may turn either to the results on the domestic fowl and its typhoid organism, *Shigella gallinarum,* or to the mouse and its typhoid organism, *Salmonella*

typhimurium. The host, through genetic means, may be differentiated into pure-breeding strains each with characteristic resistance to a given line of the pathogen. In the mouse we have six such strains. The Ba and L strains have low resistance, the E and Z strains have medium resistance, and the S and RI strains have great resistance to the disease organism. The resistance differences appear due to the cellular pattern of the mouse as indicated by the blood, spleen, and liver. We have been unable to find evidence for the resistance differences being due to any humerol constituents as, for instance, agglutinins or bactericidins in the blood serum. General constitution as measured by normal duration of life is highly correlated with strain resistance to typhoid and may be a cause of the degree of resistance.

There is an interaction between the genetic constitutions of the pathogen and the host as shown by testing the different strains of mice with the different lines of bacteria. With living organisms the low virulent bacteria show a few deaths in the susceptible mice, a very few in the medium-resistant, and almost none in the resistant strain. With medium-virulent organisms there are a fair number of deaths in the susceptible mice, a lesser number in the medium-resistant mice, and a very few in the resistant mice. With the highly virulent line the deaths in the susceptible group are almost 100 per cent, are medium to high in the medium-resistant, and are low to medium in the resistant mice. If killed bacteria of the three lines are used, the same resistance levels are denoted in the different strains of mice but to get them it is necessary to use 100 times as many or more of killed organisms than of the living organisms. These facts show that the capacity for growth of the bacteria is not the only factor in this disease difference. Rather it indicates that there are different levels of endotoxin in the three lines of bacteria, each working on hosts of differing resistance. The mechanisms of these changes may be considered as follows: The three lines of bacteria may be regarded as of three distinct genotypes. Under the particular gene influence these lines develop an endotoxin within the bacterial cells. The endotoxin is such that it does not escape from the cell into the surrounding medium unless the cell itself is broken down. The endotoxin within each cell could be either different in amount for each line or different in type. As yet we have no evidence on this point. The living bacteria introduced into the host result in increasing amounts of endotoxin with the growth and death of the bacteria. The bacteria generate humerol antibodies, demonstrable through agglutination of the bacteria, in the host. These humerol antibodies probably take little part in the animal's immediate resistance for they appear too late in the course of the disease. They would, however, have the capacity to combine and neutralize a fair amount of toxin at a later date providing the animal survives. The main resistance mechanism so far analyzed appears to be the leucocytes of the blood and macrophages of the spleen and liver. These cells have affinity for the bacteria and for their endotoxins. The rapid disappearance of the bacteria in the resistant animals and the evident resistance shown in the formation of rather extensive lesions in some of the strains support this view.

Immunizing mice with killed bacteria prior to inoculation with living organisms indicates the mechanism by which artificial immunization may take place. In immunization the bacteria with the inheritance for low virulence have a low immunizing value. The bacteria for higher virulence have higher immunizing value. These facts point again to the genes in each of the bacterial lines as governing the formation of endotoxin either differing in amount or kind. On introduction into the host each bacterial line generates a characteristic amount of humerol antibodies in the host circulation. The cellular resistance of the host remains the same as or is increased somewhat over that of the unimmunized mice. When these immunized mice are inoculated with living bacteria of one of the different lines, it takes 100 or more times the number of bacteria to cause death as it would if the mice were unimmunized. The comparative death rates of the different strains of mice, however, remain as they were for the original untreated strains. The introduction of the dead bacteria into the host has made endotoxin characteristic of the particular line of bacteria. These endotoxins have resulted in the generation of humerol antibodies demonstrable through agglutination. These antibodies are of medium strength for the avirulent line of bacteria and of fair strength for the higher virulent lines. Presumably on introduction of the living organism these antibodies combine with them to agglutinate them and possibly to destroy some of the endotoxin through neutralization. The capacity for the resistance is thus increased about 100 fold. The cellular resistance mechanism likewise holds for the strain. Both the strains of mice and the lines of bacteria hold their relative positions in the immunization picture that they held in the case in which no immunization took place. It is simply that the levels of resistance are on a higher plane.

Genes differing possibly in side-chain structures, form H and O antigens and endotoxins differing in their capacities to produce agglutination or death. The antigens or endotoxins may reflect differences either in chemical structure of the genes as different antigens or endotoxins or, what now seems more likely, the capacities of the genes to produce small or large quantities of a single antigen or endotoxin. Host differences are attributable to host gene differences leading to the production of few or many of particular types of cells, i. e. macrophages; with specific capacities to destroy these bacteria or neutralize small or large amounts of endotoxin.

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GENE ACTION IN PARAMECIUM[1]

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I. INTRODUCTION

Gene action in *Paramecium aurelia* has been investigated (Sonneborn, '43a) most fully in the case of the action of the gene involved in the determination of the difference between "killers" and "sensitives." One race of this species makes the fluid in which it lives poisonous to nearly all other races of *Paramecium*. This race is thus a "killer." Killers are invariably resistant to their own poison. Races affected by this poison may be called either "sensitives" or "non-killers," for sensitives are never killers. Likewise, non-killers are never resistant. The killer character is determined by the combined presence of a dominant gene, K, and a cytoplasmic factor, kappa. Killers always have kappa; without kappa, clones are always sensitive non-killers. Killer clones also always have, in addition to kappa, gene K; but non-killers, lacking kappa, may have either K or its recessive allele, k. Thus, neither K nor k can initiate the production of kappa. Nevertheless, there is some relation between these genes and kappa, for clones containing kappa always have K and are never homozygous for k. The role of the genes is shown by observations on homozygous recessives (kk) produced by the self-fertilization of heterozygous killers (Kk plus kappa). In these recessives kappa is retained for a few fissions, during which the cells remain killers; hence, cells are killers when kappa is in the cytoplasm and K is absent from the new nuclei (though still present in disintegrating parts of the old nucleus). However, after a few fissions, kappa disappears and the clone becomes and remains permanently non-killer. Hence, kappa is not independently self-multiplying; it depends upon gene K for its maintenance and increase. The role of the genes is further shown by introducing kappa into non-killers. If the non-killer has the genotype kk, the resulting clone is still a non-killer; but if the non-killer has the genotype KK or Kk, introduction of kappa results in its maintenance and increase, yielding a clone of killers. Hence in relation to gene K, kappa acts something like a primer in a pump: some kappa is put in and more comes out. K seems to be like a pump that will not work without being primed. The action of gene K in controlling increase of kappa is thus dependent upon a cytoplasmic primer, kappa itself.

The work just summarized suggests that an understanding of the action of gene K might be acquired by a detailed investigation of its relations to kappa. An investigation of these relations is reported in a paper now in press (Sonneborn,

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'45), in which a hypothesis of gene structure and action is induced from the results of the experimental analysis. The main features of this hypothesis, however, were suggested in the first place by the results summarized above and certain other facts known much earlier; the experimental analysis was in fact designed specifically to test the hypothesis. I shall therefore take this opportunity to point out how the hypothesis was indicated by the available information and how it was put to experimental test. This will make it possible to put on record pertinent material not presented in the other paper.

II. INFORMATION LEADING TO A HYPOTHESIS OF GENE STRUCTURE AND ACTION

The hypothesis was suggested in the first place by an attempt to account for the observed striking difference between the gross genetic phenomena in different varieties of *Paramecium aurelia*. In variety 4, all characters (killers, mating types, antigens) studied show essentially the same system of determination and inheritance. In every case, a cytoplasmic factor intervenes between gene and character. The gene alone cannot initiate production of this factor, though maintenance and increase of the factor are under the control of the gene. On the other hand, the characters (mating types, antigens) studied in variety 1 are determined by genes whose action does not depend upon cytoplasmic primers. Of the other varieties of *P. aurelia* thus far studied, some show the system characteristic of variety 1; the rest show the system characteristic of variety 4.

Two reasons make it appear unlikely that this difference between the genetic phenomena in the two groups of varieties could be due to any very profound difference in the genes and their mode of action. One reason is the extremely close relationship of the varieties; the other is the apparent identity in the two groups of varieties of the cytological processes on which the genetic phenomena must depend.

The seven varieties of *P. aurelia* thus far discovered differ little, if at all, morphologically. They are so much alike that they would never have been recognized without the discovery of mating types. They are, in fact, defined simply on the basis of sexual isolation. Within each variety, every individual belongs to one or the other of two mating types which interbreed freely; but neither mating type in one variety interbreeds with either mating type in any other variety. (Recently exceptions to this rule have been discovered, but they do not essentially alter the effectual isolation of the varieties.) Clearly, the diverse varieties are so closely related that they could hardly have diverged appreciably in anything so fundamental as gene structure and action.

Likewise, the visible cytological features of the fertilization processes are identical in the different varieties. In all varieties the diploid micronuclei in each conjugant undergo two meiotic divisions and all the resulting haploid nuclei disintegrate except one. This one goes through a third (equational) division, the two products of which are the gamete nuclei. One gamete nucleus in each con-

jugant passes into the mate and fuses with the gamete nucleus that has remained in the mate. This fertilization nucleus, in which the diploid condition has been restored, gives rise by ordinary equational mitoses to diploid nuclei that develop into the new macronuclei and micronuclei. Meanwhile, the macronucleus originally present in each conjugant disintegrates into many pieces which are eventually resorbed. In the process of exchange of gamete nuclei no cytoplasm (or effectively none) is normally exchanged. Conjugation thus provides for an exchange of genes, but no cytoplasm; and the resulting genotype of the two mates must be identical because each of the two haploid nuclei that unite to form the syncaryon in one conjugant has its exact copy in one of the two that unite in its mate. The cytological features of conjugation thus provide no basis for the observed difference in genetic phenomena among the varieties.

Nevertheless, as set forth above, exchange of genes alone brings about identity of characters in the two mates in variety 1 and not in variety 4. In the latter variety, identity of characters develops only in those exceptional matings in which both genes and cytoplasm are exchanged. It would seem as if the action of a gene in variety 1 must be equivalent to the action of a gene plus its cytoplasmic factor in variety 4. This relation is emphasized by the fact that entirely comparable characters, such as mating types or antigens, depend upon a gene only in variety 1 and upon a gene plus a cytoplasmic factor in variety 4. Further, in variety 4 different characters depend upon different cytoplasmic factors: each cytoplasmic factor is related to a particular gene. It seems improbable that a character, such as an antigen or a mating type, could in closely related varieties be the result of biochemical materials and processes so diverse that, in one variety, a gene alone is capable of controlling them while, in another variety, the gene requires in addition a cytoplasmic primer. These reflections forced me to consider the possibility that each gene in variety 1 includes what is distributed in variety 4 between a gene and a cytoplasmic primer. In other words, the cytoplasmic primers in variety 4 may correspond to a *part* of the gene in variety 1.

This possibility is obviously in conflict with what has been regarded as a fundamental conception in genetics: the indivisibility of the gene (see, e. g., Wright, '41). Is there any reason why separable parts of the gene might be discoverable in *Paramecium* and not in other kinds of organisms? The answer, it seems to me, is provided by consideration of the peculiar nuclear conditions characteristic of the ciliated Protozoa. Only in these organisms does there exist a distinction within each cell between a physiologically functional and a physiologically non-functional nucleus. The macronucleus is indispensable and controls the physiological activities of the cell; but the micronucleus is not essential: clones live and multiply well without it and maintain their genetic characters. Consequently, loss of physiologically important parts of the micronuclear genes would be of no importance to the cell. Hence the nuclear conditions in the ciliated Protozoa are such that loss of physiologically active parts of the micronuclear genes is a theoretical possibility. In other kinds of organisms such special nuclear

conditions do not exist and there is consequently no opportunity for gene disintegration to occur with impunity and so to be capable of detection.

By the same reasoning, however, one may conclude that the physiologically active macronucleus should in general retain the complete genes, for disintegration of these would be fatal to the organism. It would seem, therefore, that no essential difference should exist between the macronuclear genes in different varieties of *P. aurelia*. If the cytoplasmic primer in variety 4 corresponds to a part of the gene in variety 1, it should exist as a part of the macronuclear genes in both varieties.

This leads at once to the question of how the macronuclear genes could acquire their primer parts. There is no difficulty in variety 1 because both parts of the gene occur in the micronuclei which transform directly into macronuclei after fertilization. In variety 4, on the other hand, the micronuclei that give rise to macronuclei lack the primers. Previous experiments (Sonneborn, '43a) have shown, however, that the primers are in the cytoplasm at the time of fertilization. The new macronuclei at the time of their origin are therefore surrounded by cytoplasm containing the primers and could obtain them from this source. Further, the old macronucleus always disintegrates prior to the formation of new macronuclei, thus providing the cytoplasm with primers for the latter. The visible cytological processes accompanying fertilization thus supply a mechanism for the transfer of primers from macronucleus to macronucleus in those varieties in which the micronuclear source is cut off.

Here then is a clue to a mechanism of priming in variety 4. Gene K is the micronuclear gene which is normally transferred through the gamete nuclei at conjugation. In this form it is self-multiplying, both in micronucleus and macronucleus. If it unites, in the macronucleus, with kappa obtained from the cytoplasm, the complete gene is constituted—comparable to the micronuclear genes in variety 1—and undergoes self-duplication in the complete form. The necessity for priming arises from the separation of the parts of the gene and the inability of one part of the gene to produce the other part. In effect, K and K plus kappa are alleles and the change from one to the other is a mutation. The primary action of a gene, on this view, is self-duplication: K controls the production of kappa by reason of the fact that kappa becomes a part of the gene and thereby is reproduced as a part of it.

III. TESTS OF THE HYPOTHESIS

The preceding considerations seemed to justify the adoption, as a working hypothesis, of the assumption that K and kappa are capable of union and are in fact united in the macronucleus of killers. This hypothesis was subjected to the experimental tests presently to be set forth. To understand the tests, however, it is necessary first to recall certain important features of the macronucleus.

As set forth above, the macronucleus arises from the syncaryon as a simple diploid nucleus. It then grows enormously, becoming a multiple nucleus containing at least 30 units, each with a complete diploid set of genes. At each fission

the macronucleus divides amitotically, approximately half of the component unit nuclei passing to each daughter nucleus. However, the unit nuclei themselves must (on the basis of genetic evidence) divide by some sort of mitotic process, though the two products of division of a unit probably pass as a rule to the same daughter macronucleus. At times of fertilization, the compound macronucleus falls apart into its component units and these are resorbed in the cytoplasm.

With these features of the macronucleus in mind, the hypothesis that K and kappa are combined in the macronucleus could be tested if there were available a method of varying the amount of kappa introduced into non-killers containing gene K. For the K genes of the macronucleus may be considered as specific receptors for kappa and, if small enough amounts of kappa are presented to a macronucleus containing many K genes, there should not be enough to combine with all of these genes. Consequently, the amitotic divisions of the macronucleus during the course of repeated fissions would have to yield eventually some macronuclei completely devoid of kappa and some completely saturated with kappa. The former therefore could not yield killers, while the latter could.

The required method was developed by taking advantage of the following observation. When killers are crossed to non-killers, normally no cytoplasm (or effectively none) is exchanged and the conjugant pairs separate quickly after fertilization is completed: less than 3½ minutes elapse from the beginning of the separation process (at the anterior ends) until it is completed (in the region of the peroral cones across which the migratory gamete nuclei pass during fertilization). In the exceptional cases in which more than 30 minutes is involved from the beginning to the end of the separation process, cytoplasm is invariably exchanged and in amounts sufficient promptly to transform the non-killer mate into a killer. When the separation process takes an intermediate time, intermediate results are obtained presumably because intermediate amounts of kappa pass from the killer to the non-killer mate. This then provides a method of introducing reduced amounts of kappa into KK non-killers. However, the kappa is introduced at the time of fertilization and therefore before the syncaryon has produced the simple diploid nuclei from which the new macronuclei are to arise. Kappa is consequently present in the cell at the time the presumptive new macronuclei are in the simple diploid condition. Entrance of kappa into them at this time would result in macronuclei saturated with kappa. The results now to be set forth, however, indicate that the macronuclei may fail to become saturated. It appears, therefore, that kappa, when present in the cytoplasm in small amounts, does not necessarily get to the K genes of the new macronucleus before the latter begins to acquire its multiple condition. For the clones developed from such conjugants showed precisely the predicted segregation of kappa during the course of vegetative reproduction: lines of descent totally lacking kappa arose within these clones after from one to nearly 90 successive fissions, in different instances. Kappa, then, is unequally divided at fission in agreement with the amitotic division of the macronucleus.

While the preceding result was predicted on the basis of the hypothesis, the character of the lines which retained kappa (and the great majority did) was totally unexpected: they failed to become killers during long periods of asexual reproduction. That kappa was being produced and maintained all this time was nevertheless clearly demonstrated both by the ability of these non-killers to transmit kappa to other cells during conjugations involving cytoplasmic exchanges and by the fact that they usually yielded 100 per cent killer progeny when they underwent self-fertilization.

The 100 per cent maintenance of the non-killer character during vegetative reproduction and the 100 per cent transformation into killers after fertilization, while not foreseen and predicted, finds a simple explanation on the hypothesis under analysis. It is in fact precisely what would be required on this hypothesis, if the kappa combined with K in the macronucleus is unable to get back into the cytoplasm from the intact macronucleus, and if the killer character depends upon the presence of kappa in the cytoplasm. Under such conditions the non-killer character of the lines that maintain kappa is due simply to the combination of all the available kappa with K genes in the macronucleus leaving none (or effectively none) for the cytoplasm. The transformation of these non-killers into killers after fertilization would follow from the great excess of the kappa released into the cytoplasm at the time the compound macronucleus disintegrates, over the relatively small amount needed to saturate the K genes of the simple diploid or slightly compound macronuclei when they first develop after fertilization. As a consequence of the great disparity between the amount released by the many K genes of the old macronucleus and the amount with which the few K genes in the new macronuclei can combine, much is left over for the cytoplasm and the cell gives rise to a killer clone.

The observations on the consequences of introducing very small amounts of kappa into KK non-killer cells that previously lacked kappa are thus in agreement with the proposed hypothesis that K and kappa are united in the macronucleus when both are present in a cell. The observations have further indicated (1) that the kappa thus combined with K in the macronucleus does not escape from the intact macronucleus into the cytoplasm; and (2) that the phenotypic action of kappa depends on its being present in the cytoplasm. These ideas could be tested in another way if there were a method by which the number of K genes could be greatly increased without a corresponding increase in the amount of kappa. For this should lead by a different route to the same result as that obtained in the preceding experiments: in both cases the situation would be such as to yield some K genes lacking kappa.

This type of experiment is possible by taking advantage of the following known facts. At the time of fertilization, the old macronucleus, as has been said, breaks down into 30 or more pieces each of which contains at least one full diploid set of genes. Furthermore, the preceding experiments showed that KK non-killers containing kappa have kappa in the cytoplasm at this time. If, as the

experiments indicated, the kappa was previously combined with K in the macronucleus, the kappa must be released into the cytoplasm when the macronucleus disintegrates into pieces. During the course of the next two cell divisions, the new macronuclei which have arisen from the syncaryon presumably take up kappa. While the new macronuclei are growing and developing their normal compound condition, the pieces of the old macronucleus also grow though they are destined soon to be resorbed in the cell. This growth of the pieces of the old macronucleus involves at least a four-fold increase in volume during these first two cell divisions. This suggests that the genes of the macronuclear pieces undergo some multiplication after they have released kappa and before they are resorbed. At this stage, it is possible experimentally to suppress the division of the new macronuclei so that at the second postzygotic cell division some of the cells fail to get macronuclei. The pieces of the old macronucleus, however, are passively distributed (without division) among the daughter cells, so that each of the four cells gets about one-fourth of the pieces. In the cells that lack macronuclei but possess pieces of the old macronucleus, the latter not only fail to be resorbed, but each piece regenerates into a complete compound macronucleus (Sonneborn, '40, '42). The pieces are distributed at random during subsequent fissions until there is only one per cell, and thereafter this one, which by that time has reached full macronuclear size, divides normally at subsequent fissions. In this way, therefore, it is possible to get the many K genes of the pieces of the old macronucleus and the K genes they produced after they lost kappa to become functional and hence again to be receptors for kappa. In other words, many K genes normally destined to be lost are retained and multiplied and so provide a greatly increased number of kappa receptors.

This technique of increasing the number of receptor K genes was applied to animals of the pure killer race 51 in which non-killers had never before been found, in order to discover whether the increase in number of K receptors for kappa would yield any K genes lacking kappa. As in the previous experiments, macronuclear amitosis should eventually yield lines lacking kappa if any of the K genes of any of the regenerated macronuclei lacked kappa. The experiment did in fact yield both non-killers containing kappa (as in former experiments with introducing small amounts of kappa into KK non-killers) and non-killers from which kappa disappeared permanently, never reappearing in the course of subsequent vegetative or sexual reproduction. The experiments involving increase of K in killers thus leads to the same results as introducing small amounts of kappa into KK non-killers; both types of experiments yield the results predicted on the hypothesis that kappa is a dissociable part of gene K.

IV. DISCUSSION

A. *Darlington's views on the action of genes K and k.*—Darlington ('44) has suggested that kappa is not maintained by K, but is either inactivated by k

or is overgrown by a competitive plasmagene controlled by k. It has been shown above, however, that a pure killer race can be induced to yield a pure non-killer branch from which kappa permanently disappears without the introduction of k or any product of k into the organisms. This result renders Darlington's suggestion unnecessary and unlikely; and the means employed in the experiment to bring about the result support the alternative interpretation that K controls the increase of kappa. In a previous paper (Sonneborn, '43b) loss of killing action was reported in another race (47), and Darlington attributed this to hybridization with a race containing k. However, race 47 was isolated from all other races in the laboratory and had never been hybridized with another race during the years it was a killer or for a long time after it had become a non-killer. Nor has it at any time given any indication of containing gene k. In the absence of any evidence for Darlington's suggestion as to the role of k and, particularly, in view of the contrary evidence for the action of gene K, his interpretation seems unacceptable.

B. *Some unsolved problems.*—The major problem that remains unsolved is to account for the increase of cytoplasmic kappa. The material in sections II and III provides an explanation of how kappa primes K to produce more kappa in the macronucleus; but no data and no suggestions have yet been given as to how kappa is multiplied in the cytoplasm. It is known only that increase of kappa in the cytoplasm (which must occur in killer clones) depends upon the presence of K (and kappa?) in the macronucleus. The simplest suggestion appears to be that increase of cytoplasmic kappa may be brought about in essentially the same way as is the increase of macronuclear kappa, i. e., by combination with K, a part of K, or a product of K which, unlike kappa, is capable both of passage from the intact macronucleus to the cytoplasm and of very limited self-duplication in the cytoplasm. On this, however, there is as yet no experimental evidence.

Many other questions also need to be answered. Do the genes of variety 1 contain primers, as the hypothesis holds? Can alleles differ in the primer component only, i. e., can more than one kind of primer combine with the same basic part of the gene? If so, can two or more kinds of primers combine simultaneously with the same basic part? Is the primer alone the active part of the gene, or is the physiological specificity of the gene partly dependent on the basic gene also? These questions and others are now under investigation.

C. *Gene structure and action.*—The present paper presents and tests the hypothesis that the gene consists of two parts. One part, K in the example analyzed, is found in the chromosome, though its occurrence or the occurrence of part of it in the cytoplasm has not been excluded; the other part, kappa, occurs in both the chromosome and the cytoplasm. K can exist and multiply without kappa, but kappa cannot long exist or multiply without K. K alone produces no detected phenotypic effect; but kappa determines the killer phenotype. The possibility still remains that K not only controls the multiplication of kappa, but also, when in the presence of kappa, plays a part in the determination of the phenotype. The main features of gene action appear to be (a) self-duplication, and

(b) providing the cytoplasm with replicas of itself or part of itself. No effect of the gene on the phenotype is detected when the complete gene is confined to the macronucleus; only when at least kappa is also in the cytoplasm is phenotypic action discernible.

That the gene may consist of two diverse parts was suggested by Correns ('19) to account for variegation and by Thompson ('25, '31) to account for the Bar phenomena. In neither case was it possible to follow separately the assumed two parts of the gene, and the hypotheses were in the main formal and not subject to experimental test. That a primary action of the gene is the liberation into the cytoplasm of complete or partial copies has been suggested by Koltzoff ('35), Wright ('41) and others. This view, although based on weighty general considerations, has also not been subjected to experimental tests.

The work here reported on *Paramecium* supplies experimental tests indicating both the bipartite structure of the gene and the identity between an active cytoplasmic component and a part of the gene. The data, especially when they are presented in detail, need to be critically examined to see if there can be suggested some satisfactory alternative to the conclusion that K combines with kappa in the macronucleus. I have been unable to find one, but others may succeed where I have failed. In the absence of such an alternative, the present work may serve as a beginning of an experimental approach to the difficult problems of the structure and primary action of the gene.

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SPONTANEOUS MUTATIONS OF BACTERIA

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The study of bacterial mutability is our only avenue of approach to problems of heredity in bacteria. This approach may be followed along two lines. Along one line one focuses his attention on the phenotypic differences between two strains which differ by one mutational step, in the endeavor to trace the reaction chains from the phenotype back to the gene. This is the analogue to physiological genetics and is exemplified, for instance, by E. H. Anderson's (1) comparative study of the physiology of virus-resistant bacterial mutants.

Along the other line one focuses his attention not on the mutant and its phenotype but on the mutational step, specifically on the rate at which it occurs under some standard conditions. At first sight such a study seems rather aimless because the use of determining this or that mutation rate is not apparent. Studies of this kind can, however, become of interest in two ways. It may be found that the mutation rate can be controlled by cultural conditions. Such a finding might give us a lead as to the nature of the mutational process. Investigations of this sort have not yet been reported in the literature but very likely could be undertaken with great profit. Another use of the study of mutation rates lies in the possibility of considering the mutational pattern of a strain of bacteria and of its mutants. Attempts along these lines have been reported by Luria (2), by E. H. Anderson (1), and by Demerec and Fano (3). It is quite evident from these papers that the same mutational step can be identified in organisms which differ by one or more mutational steps of a different kind. This use of mutation rates, and of mutational patterns, may prove to be an invaluable substitute for Mendel's experiments for purposes of factor analysis in bacteria.

The main problems which face the experimenter along this line are:

1. The estimation of the number of mutants in a given culture.
2. The evaluation of the mutation rate.
3. The differentiation between mutations which may lead to phenotypically similar forms, and conversely the identification of the same mutation if it occurs repeatedly.

Before discussing these topics, I would like to make a few general remarks about methods of detecting and culturing mutants of bacteria. Some of these remarks are also pertinent to the study of mutations of viruses.

1. POPULATION DYNAMICS AND MUTATION PATTERNS

We will assume that we start from a stock strain with fairly constant properties. Actually, a variety of mutations will occur during the subculture of such

a strain. The strain will, therefore, not be pure in the genetic sense, but will represent a population which is in approximate equilibrium with its mutants. The equilibrium will be determined by three factors, namely, the forward mutation rates, a_1, a_2, etc., which lead away from the normal type, the reverse mutation rates, b_1, b_2, etc., and the selection rates, s_1, s_2, etc.[1]

We will measure mutation rates in units of mutations per bacterium and per time unit, and selection rates by the difference between the growth rate constants[2], k_N, of the normal type, and k_M, of the mutant type,

$$s = k_N - k_M$$

The populational equilibrium between the normal type and its mutants will depend on the cultural conditions. If the cultural conditions are altered the equilibrium will in general shift, and the shift may or may not be reversible when the strain is returned to the standard conditions.

Certain conditions must be fulfilled by the mutation and selection rates for the equilibrium to be reasonably stable. For instance, if a certain forward mutation rate, a_1, is high, it must be balanced either by a high selection pressure against this mutant, or by a high reverse mutation rate.

If the mutant is balanced by adverse selection then the equilibrium proportion of mutants is given by[2]

$$M/(M + N) = a/s$$

and this fraction must be small compared to unity. If a sub-culture is started from a single normal bacterium, equilibrium will be approached with a rate equal to $s - a$. Since the mutation rate must be small compared to the selection rate, the approach to equilibrium is essentially determined by the selection rate.

If the mutant is balanced by reverse mutation then the equilibrium proportion of mutants is given by

$$M/N = a/b$$

Since this ratio must again be small, the reverse rate must be large compared to the forward rate. The rate of approach to equilibrium in this case is determined by the sum of the forward and reverse mutation rates, $a + b$, that is, essentially by b.

If the forward mutation rate is very small it need not be balanced by selection pressure against it or by reverse mutations because the mutant type will in general be eliminated on each sub-culture when small samples are used for the sub-culture.

It may sound strange that the conditions of equilibrium should differ for small and high mutation rates. Actually, of course, there is no difference in the

[1] Strictly speaking, we should also include among the factors which determine the population equilibrium all the rates of mutation of the mutants. However, most of these second-step mutations will cause only small shifts of the equilibrium. If a particular step should be fast the two steps can be lumped together as one mutation.

[2] See Appendix I at the end of this paper for definition of growth-rate constants and for mathematical derivations.

equations which determine the average values of mutants in a very large number of cultures in these two cases. However, for small mutation rates the fluctuations in these numbers become the dominant feature. Suppose we are dealing with a mutation rate of 10^{-8}. If we were dealing with millions of similar cultures, or if we would sub-culture one culture many million times we would be likely at one time or another to transfer a great many mutants, and from then on the culture would contain a high proportion of mutants. In actual experiments, however, we are not likely to encounter these rare events which are included in the ideal average.

When studying the mutational pattern of a stock strain with reasonably stable properties we may therefore expect to find, on the one hand, mutants which occur with high frequency but grow more slowly than the normal type or revert quickly, and, on the other hand, mutants which occur with low frequency and which may or may not be at a selective disadvaneage due to slow growth or reverse mutations. These expectations are borne out by the general experience that stable and vigorous mutants of stock strains always occur with very low frequency.

The same is not true of the mutational pattern of the mutants themselves. The only prediction that can be made regarding the patterns of the mutants is that mutants which occur frequently and are not at a selective disadvantage must have a high reverse rate. Such mutants are, of course, of great importance for the study of the mutational pattern of the normal type, but it stands to reason that their study presents special difficulties.

2. THE ESTIMATION OF THE NUMBER OF MUTANTS

We may distinguish between three methods of detecting mutants in cultures of bacteria.

The first method consists in plating out a sample of the normal culture so as to obtain isolated colonies. The mutants may then be picked out either simply by inspection of the colonies or by special tests of a large number of colonies selected at random. The first alternative is used for picking mutants which affect the morphology or pigmentation of colonies. Using the technique of Spiegelman, Lindegren and Hedgecock (4), it may also be found possible to pick by this method mutants which affect the fermentative properties of the strain. The second alternative has been applied by Roepke, Libby and Small (5) to obtain growthfactor-deficient mutants.

The second method is to subject the normal culture to a destructive treatment, such as irradiation with ultra-violet light (Maisel-Witkin unpublished) or exposure to penicillin (Demerec unpublished). If the normal population contains mutants with somewhat heightened resistance to these destructive agents, then these resistant mutants will be present in higher proportion in the fraction of bacteria which survives the treatment. In the ideal case the surviving fraction of bacteria will consist entirely of mutants with increased resistance. The method

amounts, in such a case, to a direct isolation of the mutants, but of course not all mutants with increased resistance present in the original culture are isolated by this method, because most of them will be killed by the treatment. Consequently, it is quite difficult to determine the fraction of this mutant type in the normal culture.

The third method is to use a treatment which will eliminate all normal bacteria while leaving all individuals of certain mutant types unaffected. The only treatment known that will give such a clear-cut segregation of certain mutant types is exposure to an excess of virus particles which are active on the normal strain of bacteria. Luria has shown that a similar principle can be used to isolate mutants of *viruses,* by plating a large number of virus particles with a strain of bacteria resistant to the normal type of virus of the stock used. Such stocks may contain mutant viruses which will attack and multiply at the expense of the bacterial strain.

Mutants must fulfill certain conditions to permit their maintenance and study. In general, reverse mutation rates must not be so high that the normal populational equilibrium is reached in a single sub-culture. In growth factor mutants the conditions are more stringent because even a very low reverse mutation rate will invalidate the test of mutation.

In virus-resistant mutants the conditions for maintenance are less stringent. Here the reverse mutants are automatically eliminated if plating is done in the presence of excess virus. If the reverse rate is rather high the mutant may become extinct in colonies starting from a single resistant bacterium. The chance for this to occur is $b/(1 - b)$, where b is the reverse rate per generation. The proof for this statement is given in Appendix II.

3. MUTATION RATES

In the past there has been much confusion about the concept of mutation rate. Many authors have simply divided the number of mutant bacteria found in a culture into the total number of bacteria and have called this fraction the mutation rate. It is obvious that the fraction of mutants is a poor measure of the mutation rate, since the number of mutants depends on two factors, namely, the number of mutations occurring in the culture and when they occurred. A mutation which appeared several generations back will be represented, at the time of the counting, not by one mutant, but by a sizeable clone of mutants.

First of all, we must define a mutation rate in such a fashion that it becomes a characteristic of each bacterium and expresses a property of this bacterium which can be measured (6). We define the mutation rate, therefore, as the probability of a bacterium to mutate during a given time unit under some specified physiological conditions. The time unit, of course, must not be greater than the lifetime of the bacterium. This conception of a mutation rate is quite analogous to the concept of probability of decay of a radio-active atom during a time unit.

In a growing culture of bacteria, mutations will occur with a frequency per time unit which increases in proportion with the size of the population. At any arbitrary moment, therefore, various-sized clones of mutants will be present in the population. Compare, for instance, clones of size 64 with those of size 128. Clones of these sizes originated in mutations which occurred respectively 6 or 7 generations back. Six generations back twice as many normal bacteria were present as seven generations ago. Clones of size 64 should therefore be twice as frequent as those of size 128. On the other hand, the average number of *mutants* belonging to clones of size 64 should just equal that belonging to clones of size 128. By logical extension one finds that the average number of mutants in a culture, say, 30 generations old, should fall into 30 groups of equal size, each group containing mutants of the same clone size. This statement, while correct, is not applicable to any real experimental situation, because the large clones, which contribute a large portion to the average, are so rare that they will not likely be found in a limited number of trials. The likely average number of mutants in a culture must therefore be calculated with omission of the contributions from the large but rare clones.

The likely average fraction of mutants thus turns out to be smaller than the ideal average fraction. The ideal average fraction would be equal to the rate multiplied by the time since the start of the culture. The likely average, on the other hand, is obtained by measuring the time, not from the start of the culture but from the time when the culture had reached a size at which a mutation becomes likely. Thus, for a mutation rate of, say, 10^{-8} per generation, we should count the time from the moment that the culture has reached a population size of about 10^8. If our observation is made when the population has reached a total number of 10^9, the time to be used would be 10, instead of 30, the number of generations since the start of the culture. Our correction in this case amounts to a factor of three. If the mutant grows more slowly than the normal type we must use a further correction to take into account that the mutants have not multiplied at the same rate as the normals.

The determination of the mutation rate from the number of mutants present at any given time suffers from another complication, namely, the very large fluctuations of the number of mutant bacteria in a series of similar cultures. It is easy to see why the fluctuations must be large. As we have seen above, the mutant bacteria, on the average, stem in equal numbers from all preceding generations. If the mutation rate is small, mutations will occur with any reasonable probability only during the last few generations, say the last ten generations as in the example above. Now the quantity which is subject to normal fluctuations is not the number of mutants but the number of mutations, in each generation. In the example cited one-tenth of the mutants will, on the average, be due to one mutation which occurred ten generations back, another tenth will be due to two mutations which occurred nine generations back, etc. It is clear that the one and two, etc., mutations are subject to large fluctuations. The net fluctuation in the final

number of mutants is therefore very appreciable, even if the number of mutants is quite large.

To make matters worse, the customary recourse to using large numbers of similar cultures for determining the average number of mutants is here of little use, because the more cultures we use the more likely we are to have a mutation occurring at a very early stage in the development of one of the cultures. Such an event is like hitting the jackpot; it will give us an erratic number of mutants and a most undesirable increase in the fluctuations.

To sum up this matter, we may say that the determination of the mutation rate from the number of mutants, even if we can measure the number accurately, is necessarily a statistically inefficient procedure.

4. REVERSE MUTATIONS

I should like to add a few remarks about the problem of studying reverse mutations. As has been pointed out before, reverse mutations, even if they occur frequently, do not interfere with the detection of forward mutations in mutations which affect the reaction of bacteria to viruses. While this is an advantage to the study of the forward rate it is a decided disadvantage in determining the reverse rate. At present we do not know of any generally applicable method for the determination of mutation rates from virus resistance to virus sensitivity.

Just the opposite of what has just been said is true of mutations which involve the acquisition of growth-factor requirements. These can be discovered only if the reverse rate is exceedingly small. However, if the reverse rate is small enough to permit the cultivation of the forward mutant then the reverse rate, even though it be very small, can be measured with reasonable accuracy. This is quite apparent from the work of Roepke, Libby and Small (5). Their method of detection of reverse mutants is to culture the factor requiring mutant in a basal medium to which a sub-optimal amount of the growth factor has been added. If reverse mutation occurs it shows by delayed full growth in this medium.

Now the mutants selected by any of the methods mentioned may differ from the normal in more characters than the one used to select it. Specifically, E. H. Anderson (1) has shown that many of the virus-resistant mutants are also mutants deficient in growth factors. In these special cases, therefore, the two methods might advantageously be combined; that is, one may use the virus resistance for the isolation of the forward mutants and the growth factor requirements for the detection of reverse mutation.

5. IDENTIFICATION AND DIFFERENTIATION OF MUTANTS

The chief interest of a study of a hypothetical pair of forward and reverse mutations would lie in ascertaining whether or not true reverse mutations do occur. It is difficult to decide whether the revert strain is identical with the original one. Theoretically, this implies the comparison of the original with the presumed revert strain in all measurable characteristics. In practice one has to

restrict himself to a selection of some fairly comprehensive tests. Tests with viruses are among the easiest.

The following example may serve as an illustration of the method. Two mutant strains of a bacterial strain (*E. coli* "B") had been isolated, one by using virus T1 as selecting agent, the other by using T5. These strains may be referred to as B/1 and B/5, respectively. Each of these strains was then plated with seven different viruses, T1 to T7, and with each virus they were tested in two ways, once by plating a large number of the bacteria (about 10^8) with an excess of virus particles, and once by plating with about 100 virus particles. The results are given in Tables I and II.

TABLE I

PLAQUES OBTAINED WHEN PLATING LOW TITER STOCKS OF VIRUSES ON B/1 AND B/5

	T1	T2	T3	T4	T5	T6	T7
B/1	0	183	118	146	0	47	59
B/5	0	160	106	172	0	44	64

Table I gives a comparison of the plaque counts obtained with these two bacterial strains for the seven viruses. It will be seen that both strains are resistant to both T1 and T5, and that they give equal plaque counts with all the other viruses. The two strains, therefore, agree in their resistance pattern.

At this point, however, we must inquire somewhat more closely into the nature of the mutations which make bacterial strains resistant to one or another group of viruses. It used to be believed that these mutations fall into two classes: On the one hand, there were thought to be mutations which completely alter the sensitivity pattern of the bacterial strain, and which in general also alter the morphology or other characteristics of the colony of the strain. These are the well-known "dissociative" changes from smooth to rough, etc. On the other hand, there were, it was believed, highly specific changes towards resistance to only one, or to a group of closely related viruses. These conceptions are due chiefly to Burnet (7), and the idea of classifying viruses by cross-resistance tests goes back to an important paper by Bail (8).

However, recent work has indicated that these conceptions are not tenable (9). There is no clear-cut distinction between mutations with generalized effects and those which cause nothing but resistance to a group of similar viruses. Specifically, it has been found that the classification of viruses by cross-resistance tests does not at all lead to a satisfactory grouping. Such classifications bring together viruses which are quite unrelated on other criteria, and they separate viruses which, on all other criteria, are most intimately related. The most important among these other criteria are the morphology as revealed by the electron microscope and cross-inactivation tests with specific antisera.

On second thought, we should not be surprised by these findings. Once it is granted that the mutations in question are not induced by the virus but are spontaneous, it seems understandable that one mutation may affect the sensitivity of the bacterium to quite unrelated viruses. At present, we do not know what makes a bacterium sensitive or resistant to a virus. We can imagine that certain substances must be elaborated whose presence permits the virus to grow in the bacterial cell. It stands to reason that a variety of genetic changes could interfere with the network of synthetic processes in such a fashion that the bacterium becomes unsuitable for the growth of the virus. Furthermore, resistance to two unrelated viruses in this picture means that there is a tie-up between the synthetic chains which lead from a gene to the substances required by these two viruses, respectively. The viruses themselves need have no similarity with each other. These ideas have been explained more fully in a recent paper by E. H. Anderson (1).

While we thus lose the cross-resistance test for the classification of the *viruses*, we can still retain the virus-sensitivity tests for the classification of *bacteria*, as in Table I. Tests of this kind have been used for the classification of naturally occurring strains of many species of bacteria. On the whole, the "typing" of an unknown strain by its virus-sensitivity pattern seems to be about as satisfactory a method as any other, and it seems to agree with serological typing.

Here, however, we are more interested in the possibility of classifying, not arbitrarily selected wild strains, but the family of mutants of one strain. With how much assurance can we here recognize identical mutants by their sensitivity pattern? An important piece of evidence on this point comes from the work of Demerec and Fano (3). These authors find that the same sensitivity pattern may be reached either in two mutations or in one mutation. Is the end product here genetically alike in the two cases, or only phenotypically? If the first, we would have to assume the existence of coupled mutations; if the latter, we might hope to identify phenotype differences by further tests, either with other viruses, or by physiological studies. Until such studies have been carried much further we can not form an opinion on the merits of the sensitivity tests for purposes of classification.

TABLE II

RESISTANT COLONIES OBTAINED WHEN PLATING HIGH TITER STOCKS OF VIRUSES ON B/1 AND B/5

	T1	T2	T3	T4	T5	T6	T7
B/1	Inf	4	233	275	Inf	38	518
B/5	Inf	14	200	250	Inf	20	600

Table II gives the number of resistant colonies obtained when plating these two strains with any of the seven viruses. Both B/1 and B/5 give complete growth when plated with high titers of T1 or T5, since they are resistant to these viruses. With the other viruses they give a fairly characteristic number of mutants, and it will be seen that the number of mutants obtained from B/1 is in all cases quite similar to the corresponding number obtained from B/5. What we are here comparing is really a portion of the mutational pattern of these two strains, and the parallelism might be considered strong evidence for the similarity, if not identity, of the strains.

Again, however, the results obtained by Demerec and Fano (3) should make us cautious before accepting this similarity of the mutational pattern as evidence of genetic similarity. In very extensive tests these authors could show that the mutational pattern is remarkably similar in bacterial strains which differ by one or two mutational steps. Similar results were found by Luria (2) and by E. H. Anderson (unpublished). Since, therefore, the available evidence shows that mutational patterns may be similar in strains with known differences, we cannot accept the similarity of the mutational pattern as a criterion of genetic identity.

MATHEMATICAL APPENDIX

I. Population dynamics.—

We wish to prove in this Appendix some of the quantitative statements made in the text. This will help to clarify their meaning and the limits of their applicability. The results are probably not new, the proofs perhaps somewhat simpler than those given previously. All equations refer to average values and are therefore of practical value only for the very high mutation rates where the fluctuations mentioned in the text are not the dominant feature.

Notation:

N = number of normal type bacteria at time t.

M = number of mutant type bacteria at time t.

a = forward mutation rate, defined as the fraction of normal type bacteria mutating per time unit.

b = reverse mutation rate, defined as the fraction of mutant type bacteria reverting to normal type per time unit.

k_N = growth rate constant of normal type bacteria, defined as the fraction of normal type bacteria which divide per time unit.

k = growth rate constant of mutant type bacteria, defined as above.

$s = k_N - k_M$ selection rate of normal against mutant type.

From these definitions follow the basic equations:

$$dN/dt = (k_N - a)N + bM$$

$$dM/dt = (k_M - b)M + aN$$

Case a.	*Case b.*
Forward mutation balanced by selection, no reverse mutation $b = 0$	Forward mutation balanced by reverse mutation, no selection $s = 0$, $k_N = k_M = k$

For these special cases the equations (1) simplify, respectively, to:

$$dN/dt = (k_N - a)N \qquad dN/dt = (k - a)N + bM$$
$$dM/dt = k_M M + aN \qquad dM/dt = (k - b)M + aN$$

By changing to logarithmic derivatives and simple transformations these equations are easily transformed to:

$$d(M/N)/dt = +a - (s - a)M/N \quad | \quad d[M/(M+N)]/dt = a - (a+b)M/(M+N)$$

The following integrals of these equations are adjusted to satisfy the initial condition $M = 0$ at $t = 0$:

$$\frac{M}{N} = \frac{a}{s-a}\left(1 - e^{-(s-a)t}\right) \qquad \frac{M}{M+N} = \frac{a}{a+b}\left(1 - e^{-(a+b)t}\right)$$

In both cases the equilibrium values given in the text are found by putting t equal to infinity.

$$\left(\frac{M}{N}\right)_{equ.} = \frac{a}{s-a} \qquad \left(\frac{M}{M+N}\right)_{equ.} = \frac{a}{a+b}$$

II. Probability of extinction of a type which mutates with a frequency "a" per generation, without reverse mutation.—

Let us assume that we start a culture with one individual of the normal type. The normal type may then become extinct by mutation of this individual before it divides; or, it may become extinct after the first division, if both offspring mutate before they in turn divide; or, if one of them mutates and the other divides and both its offspring later mutate, and so on.

We may ask what the total probability is that the clone generated by one bacterium will eventually contain none of the original type. It is obvious that the original type is certain to die out if the mutation rate is greater than .5 per generation, because then the net growth rate of the type is negative; that is, on the average the type loses more individuals by mutation than it gains by reproduction in each generation. The probability of extinction is therefore 1 for a mutation rate of .5 per generation, or any greater mutation rate.

However, also for mutation rates smaller than .5 per generation the type may die out by chance during the early stages of reproduction. The total chance may be calculated by a very simple argument. We wish to calculate the chance of extinction of the normal type in a clone which starts with one bacterium of the normal type, just after the birth of this bacterium. This chance will be a function of the mutation rate "a." Call the function $L(a)$. We calculate it by making use of the fact that any normal type offspring gives rise to its own sub-clone, and

that the chance of extinction within this sub-clone must be equal to the chance of extinction in the whole clone. This fact allows us to set up a simple equation for $L(a)$. Let us consider events up to the moment of the first division. Up to this time the chance of extinction is a, the chance of survival $1 - a$. In case of survival up to this point two normal type bacteria will be formed by division, and the chance of extinction for each of their sub-clones is again $L(a)$. The chance that both sub-clones will die out is, therefore, $L^2(a)$. The total chance that the whole clone dies out is, therefore,

$$L(a) = a + (1 - a)L^2(a)$$

This quadratic has two solutions, namely,

$L(a) = 1$ which is valid for a greater than .5

and $L(a) = a/(1 - a)$ which is valid for a smaller than .5

The complementary chance, that of survival of the type, is

$$S(a) = 1 - L(a) = (1 - 2a)/(1 - a)$$

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GENETICS OF BACTERIUM - BACTERIAL VIRUS RELATIONSHIP[1]

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VIRUS-RESISTANT BACTERIAL MUTANTS

The problem of the modifications in host-virus relationship between bacteria and bacterial viruses is pertinent to the topic of this conference in many ways. First, it involves a problem of bacterial variation, namely, the study of hereditary changes in bacterial sensitivity to viruses. Second, it involves the study of a group of virus mutations, and of their relation to mutations of the host cells. Third, it leads us to a consideration of the mechanism of virus reproduction inside the host cell, which we may consider as more or less directly bearing on the mechanism of gene reproduction, particularly in view of the possible relationship between viruses and genes, which has been the subject both of older and more recent speculation.

From bacteria sensitive to a bacterial virus one can isolate virus-resistant variants, which develop after the bulk of a sensitive culture has been destroyed by the lytic action of the virus. In most cases, these resistant bacteria do not adsorb the virus; they grow in a perfectly normal way in its presence. According to d'Hérelle ('26) these resistant bacteria would stem from cells which, upon infection by virus, had acquired an hereditary immunity to it. Several other authors (Gratia, '21, Bail, '23, Burnet, '29) have maintained that virus resistance originates by a process of bacterial mutation independent of the action of the virus.[2] In some particular cases, in which resistance appeared frequently and was associated with changes in other properties, Burnet ('29) actually succeeded in isolating resistant variants in the absence of any virus. For the most common cases, in which the resistant individuals are a small minority, detectable only after lysis of the bulk of the bacterial population, a decision on their mode of origin can be reached by an indirect method (Luria and Delbrück, '43).

This is based on the analysis of the distribution of variant bacteria in a limited series of cultures started from few normal individuals. Variants originated by mutation multiply, producing clones of mutant bacteria, and the distribution of the numbers of mutant individuals can be predicted with some approximation on the basis of an analysis of the frequency distribution of the clones of various sizes. The approximately calculated distribution can be compared with the experimental one, and from the latter a fair estimate of the mutation rate is obtained by simple formulas.

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[2] An exception may be represented by some poorly understood cases of "lysogenic" resistance (virus carried and secreted by resistant bacteria) in which the resistance would appear to be the result of the established symbiosis (Burnet and Lush, '36).

This method was applied to the study of the origin of virus-resistant variants of *Escherichia coli* strain B, showing their mutational origin independent of the action of the virus. The latter simply acts as a selective agent, destroying the sensitive cells and revealing the presence of the resistant mutants. Very low mutation rates can be detected and measured with relative accuracy; for the case of mutations to resistance to virus *a*, the rate is of the order of 10^{-8} mutations per cell per generation.

A bacterium may be sensitive to several virus strains. Resistance to one or more of these may arise without affecting the sensitivity to others (Bail, '23, Burnet, '29). In some cases, resistance to two or more viruses may regularly be associated. A systematic study of the acquisition of resistance by *Escherichia coli* B to seven viruses (Demerec and Fano, '45) proved that resistance to a group of viruses may arise as a result of either one or more mutations. Particularly important seems the conclusion that the rate of a certain mutation is the same even when the mutation occurs in strains genotypically different because of previous mutations to resistance to other viruses. This suggests that different mutations may take place in different centers, as in the case of mutations in different genes of higher organisms.

It is interesting that the mutations to virus resistance are often associated with changes in other properties, although many of the mutants appear to differ from the parent strain only in virus sensitivity. Colony type (rough or smooth, Arkwright, '24, Hadley, '27), antigenic properties (Burnet, '29), growth rate (Delbrück and Luria, '42) may be changed. Recently, Anderson ('44) found that several mutants from *Escherichia coli* B require an unidentified growth factor, present in meat or yeast extracts and not needed by the parent strain.

These associated changes are evidently the result of the same mutation causing virus resistance. Some of them may actually be different manifestations of the same structural change in the bacterial surface. On the basis of studies on the relation between antigenic properties and virus sensitivity, Burnet ('30) suggested that the surface receptors for viruses are carried by the same groups carrying the heat-stable agglutinogens of the bacteria. This conclusion was supported by the study of a "virus-inactivating agent," extracted from virus-sensitive bacteria, which proved to be one of the polysaccharide antigens of the bacterial cell (Gouch and Burnet, '34). According to Burnet's idea, the common carrier of antigenic properties and virus sensitivity has an "antigenic pattern," more stable, and a "virus receptor pattern," which can undergo smaller changes, some of them not affecting the antigenic specificity.

To explain simultaneous changes in virus sensitivity and growth-factor requirements, Anderson ('44) suggested that the normal process of synthesis of the growth factor has a common link with the process of synthesis of the virus receptor. Simultaneous acquisition of resistance to several viruses, according to Anderson, may depend on mutations affecting some common step in the synthesis of the relative receptors.

VIRUS VARIANTS ACTIVE ON RESISTANT BACTERIA

Up to this point of our story, the bacterial viruses have played the role of selective agents whose action reveals the presence of the resistant bacterial mutants. The viruses, however, can themselves undergo hereditary changes in their capacity of attacking bacteria. From some of the viruses we succeeded in isolating new strains of viruses, very similar to the normal strains and still active on the original host, but capable of attacking also bacteria resistant to the normal viruses (Luria, '45). Thus, one witnesses the appearance of a virus variant endowed with a new property, namely, "virulence" or activity toward one or several mutant bacterial strains resistant to the parent virus. Similar virus variants had been noticed by Sertic ('29), and evidence of their occurrence can be found in some data reported by Burnet (1930, see Table II, p. 655).

From the viruses α, γ, T3, T6, and T7, active on the strain *Escherichia coli* B, we have isolated in pure form a series of virus variants, called α', γ', T3′, T6′, and T7′; some of these, particularly viruses α' and γ', have been studied in detail.[3]

Virus α' and virus γ' are indistinguishable from their parent viruses in every respect but their activity on resistant bacteria. Serological tests by cross-neutralization with antisera against a normal virus and its variant fail to detect any difference. The x-ray sensitivity, related to the particle size, is unchanged; this is also true for viruses T6 and T6′. Viruses α and α', and viruses γ and γ', behave identically in their action and growth on the common host B. Adsorption rate, interval between infection and lysis, and average yield of new virus per bacterium are identical for each pair of viruses.

Virus γ' seems to be active on all B/γ strains. Virus α' attacks a group of bacterial strains, B/α_2, resistant to virus α; it does not attack several other α-resistant strains B/α_1. It is interesting to notice that the bacterial strains B/α_2, sensitive to virus α', belong to the group which was found to require a growth factor not needed by strain B (Anderson, '44). The growth-factor requirement produced by mutation is therefore correlated with resistance to virus α, but not with resistance to virus α'.

The new hosts adsorb the variant viruses at a smaller rate than do the normal bacteria B. Another difference is the yield of virus per bacterium at lysis, which is significantly smaller for the new hosts.

In some other cases the virus change also affects the activity on the original host. Virus T6′, variant from T6, is poorly adsorbed, not only by its new host B/6, but also by the normal host B, which adsorbs virus T6 very rapidly.

By submitting bacteria to lysis by the variant viruses, we isolated a series of

[3] The notation for viruses and for virus-resistant bacteria used in this paper is a combination of those used by Luria ('45) and by Demerec and Fano ('45). For example, a bacterial mutant isolated in presence of virus α, and resistant to this virus, is called B/α; a mutant from B/α, isolated in presence of virus T6, will be called B/α/6. Sub-indexes indicate different bacterial strains isolated in presence of the same virus: B/α_1, B/α_2. Viruses α and γ are called viruses T1 and T2 in Demerec and Fano's notation. It is to be hoped that a generally satisfactory notation will be agreed upon in the near future.

new mutant bacterial strains resistant to them. For viruses α' and γ', the results can be summarized as follows:

A bacterial strain resistant to a variant virus is also resistant to the parent virus. Bacterial strains resistant to a normal virus, as we mentioned above, may or may not be resistant to its variants. Mutations to resistance to unrelated viruses, normal or variants, are generally independent. Exceptions are found: for instance, a strain resistant to viruses α and α', after being lysed by virus γ', showed the presence of a mutant resistant to viruses γ and γ' but sensitive to α and α'. A mutation here produced resistance to a group of viruses and return to sensitivity to another group.

It is useful to compare the range of activity of a variant virus not only with that of its parent virus, but also with that of other viruses. Similarity of host range may conceivably be an indication of genetic relatedness, unless it is proved that it can be acquired by convergent changes in the viruses. The following scheme illustrates the situation concerning viruses α, α', and T5, the latter quite probably unrelated to the others (different particle size, no serological cross-reaction). S = sensitive; R = resistant.

TABLE I

Bacterial strain	α	α'	T5	Growth-factor requirement
B	S	S	S	−
B/α_2	R	S	S	+
B/α_1	R	R	R	−
B/α_2/α'	R	R	R	+

The scheme shows that virus α' and virus T5 have the same host range, in spite of their unrelatedness. The change $\alpha \rightarrow \alpha'$ makes the virus similar to T5 in its activity range. The scheme also shows that two bacterial strains which have acquired the same range of sensitivity by one or by two mutational steps are not identical, phenotypically as well as genotypically. The study of growth-factor requirement reveals a difference which virus-sensitivity tests alone could not detect.

VIRUS MUTATIONS AND THE MECHANISM OF VIRUS MULTIPLICATION

The virus variants are detected by putting large amounts of a normal virus in presence of resistant bacteria on a solid medium: some colonies of virus (lytic plaques) develop, containing variant virus. As in the case of the origin of the resistant bacteria, we may consider alternative possibilities. On the one hand, the virus variants may originate by mutation during the growth of the normal virus on sensitive bacteria. On the other hand, they might represent the result of an

adaptation on the part of some particles of normal virus which, when in presence of resistant bacteria, succeed in attacking them, thereby acquiring an hereditary change in activity (Sertic, '29).

If the variants arise by mutation in the course of the growth of normal virus on sensitive bacteria, they will continue to multiply like normal virus particles (at least for viruses α' and γ') producing clones of variant particles. As in the case of bacterial mutations (Luria and Delbrück, '43), this clonal grouping leads to the expectation of very large fluctuations in the numbers of variant particles found in a limited series of similar virus cultures, each originated from normal virus particles.

Since the virus grows in successive "bursts" from infected bacteria, we may expect either clones of at least the size of a full burst, or smaller clones if a bacterium infected by a normal virus particle can liberate a mixture of normal and variant viruses due to the occurrence of a virus mutation in its interior.

The grouping of the virus variants was tested, for viruses α' and γ', by counting the plaques of variant virus in series of similar cultures of the normal virus after plating each culture with bacteria resistant to the normal virus and sensitive to the variant. The results (Luria, '45) showed distributions characterized by large irregular fluctuations of the proportion of variants from culture to culture, distributions best explained by the hypothesis of a clonal grouping due to mutational origin of the variants. The resistant bacteria here act as selective agents permitting the multiplication of the mutant but not of the normal virus particles.

The experiments also showed a large proportion of cultures in which the total number of variant particles was well below the average yield of virus per infected bacterium (cultures with 1, 2, 3, . . . variant particles). These particles must derive from bacteria inside which a virus mutation occurred, and which liberated a mixture of normal and mutant viruses. In order to calculate the expected distribution of these mutants and the mutation rates, we need to know how the virus multiplies inside the bacterium: but this is as yet unknown. This consideration, however, suggests that, from the experimental distribution of the number of mutants, we may be able by the reverse analysis to get information on the mechanism of virus reproduction. The distribution will indeed be different according to the mode of reproduction. Figure 1 graphically shows some of the possible methods of virus multiplication and mutation.

In fig. 1A, the virus is supposed to multiply autocatalytically, as do bacterial cells in a culture. In such case, the distribution of the number of mutants (in clones smaller than a full burst) should be the one discussed by Luria and Delbrück ('43) for bacterial mutations.

In fig. 1B, the virus is supposed to multiply by successive replications of the infecting particle. The probability that a newly formed particle is a mutant does not affect the probability of the next particle formed in the same cell: each mutant arises by an independent event inside the cell, and the numbers of mutants

will show a Poisson distribution (always for values below the full yield of virus per bacterium).

Figure 1C assumes that each virus particle is produced by replication of the last particle formed. If one particle mutates, all the following ones produced in that bacterium will be mutants. A mutation may occur with equal probability at any time, since there is only one particle multiplying at any given moment; clones of any size between one and the full yield are equally probable, and the distribution of mutant particles will show an equal frequency of all these values.

Figure 1D assumes a combination of cases B and C, in which the multiplication proceeds by replication of either of the two last particles formed. The resulting distribution of mutants will evidently be a superposition of a Poisson distribution (mutations not followed by replication) and a constant frequency distribution (mutations followed by replication).

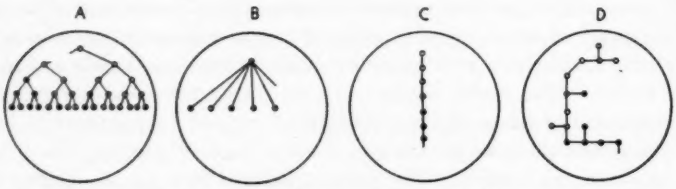

Fig. 1. Open circles = normal virus particles. Solid circles = mutant virus particles.

This type of analysis looks most promising, but unfortunately it has up to now met with a serious experimental drawback. Because of the poor adsorption mentioned above, the numbers of plaques produced by a mutant virus on the bacteria resistant to the parent virus is smaller than the true number of mutant particles by a factor generally around 0.3–0.8, but which can vary within the same experiment. The result is that one cannot obtain a sufficiently accurate experimental estimation of the distribution of the numbers of mutant virus particles. If this difficulty can be overcome, this type of analysis may prove the best way of approach to the problem of virus multiplication.

RELATION BETWEEN BACTERIAL AND VIRUS MUTATIONS

Some cases of abrupt changes (mutations) in bacterial viruses have been described before. The best-known case (Burnet and Lush, '36) concerns a virus carried in lysogenic form by a resistant staphylococcus. This virus gives a variant able to lyse the resistant bacterium from within, but still inactive on the resistant bacteria from without. A similar change was described by Gratia ('36) for a megatherium virus. In both these cases, the mutation affects the type of action of the virus on the bacterium rather than the ability to be adsorbed by and grow on resistant bacteria, as in our cases.

The fact that our virus mutants are adsorbed by bacterial mutants which do

not adsorb the parent virus focuses our attention upon the surface structures of viruses and bacteria. A change of the bacterial surface, making the cell resistant to a virus, can be compensated for by an independent change of the virus particle reestablishing the affinity of the surfaces. This fact suggests that the changes involved are small, probably consisting of simple stereochemical rearrangements. The low rate of adsorption of a mutant virus by bacteria resistant to the parent virus may be interpreted as due, either to less satisfactory "fitting" of the complementary surface structures, or to the fact that only one or few of several receptor groups on the virus surface have reacquired their affinity for the bacterial surface. It is not surprising that the antigenic properties of the viruses may not be affected by the mutations. For the virus particle, as for the bacterial receptors (Burnet, '30), we may conceive of an antigenic pattern more stable than the receptor pattern.

Given the small size and the composition of bacterial viruses (mainly nucleoprotein of the desoxyribose type), it is possible that the surface changes of the viruses actually represent the primary mutational changes. If so, the correlated changes of the bacterial surface would bear a similarity to the mutational changes in the virus. It is only a step to imagine that they are also closely related to the primary mutational changes in the bacterial cell.

The possibility that surface antigens of cells be structurally related to the genes which regulate their production has repeatedly been suggested (Irwin and Cole, '36, Haldane, '39). Sturtevant ('44) recently suggested the possibility that these genes may be reached and affected by antibodies against the antigens they determine. This possibility, offered in connection with experiments on *Neurospora* (Emerson, '44), is particularly enticing in the case of bacteria, where variation following antiserum treatment has repeatedly been reported. The surface structures involved in virus sensitivity, however, are possibly less direct products of the genes, since the same change in sensitivity is obtainable by different combinations of independent mutations (Demerec and Fano, '45; Anderson, '44). We have tried to increase the rate of bacterial mutations to virus resistance by treatment with antibacterial serum; fairly numerous attempts, however, have given as yet no indication of a positive effect.

A few words may be added on the bearing of these results on the problem of the origin of viruses. Because of their strict parasitism, it has often been supposed that they originate (possibly as "free genes" or "plasmagenes") from cells similar to those in which they can reproduce. We saw that host-virus relationship can be maintained by complementary, mutually compensating mutations. If the host mutations have evolutionary significance, as we have no reason to doubt, a virus parasitic to a certain type of cell may have derived, by a series of mutations, from a virus parasitic to a remote ancestor of its present host. Even if viruses originally derive from host cells, the genetic relationship between a virus and its present host may be very remote. The above consideration probably holds true for viruses other than bacterial viruses, since selection of virus mutants in the course of

adaptation to new hosts is probably of very general occurrence (see Findlay, '39, Burnet and Bull, '43).

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GENETICS AS A TOOL FOR STUDYING GENE STRUCTURE

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By making use of current ideas about the structure of genes it is possible to develop a formal scheme which will account for the continuity of genic specificity in gene reproduction, and for the initiation of gene-controlled reactions in the cytoplasm. Such a formulation gives a pattern into which many diverse genetic observations can be fitted and suggests definite lines of experimental approach which were not otherwise apparent.

Two characteristics of genes are important to the development of this scheme. On the one hand, genes differ greatly in their specificities as can be seen from the diverse gene-controlled reactions reported by Tatum and Beadle ('45) earlier in this Conference. On the other hand, genes are extraordinarily alike in all other respects, including their ultimate chemical constitution as understood at this time. This characterization of genes reminds one of similar properties of antibodies which resemble one another closely except in their specific relationships to their homologous antigens.

That antibody specificity resides in the unique surface configuration of a particular antibody molecule is strongly suggested by studies on the antigenic relationships of simple chemical substances (Landsteiner, '36; Marrack, '38). Pauling, Campbell and Pressman ('43) have pointed out how the specific surface of an antigen can serve as a template upon which the antibody surface is determined, the surfaces of the two molecules then being mutually complementary in shape and in the arrangement of reactive groups (i. e., oppositely charged groups, groups capable of forming hydrogen bonds, etc.).

Complementary, antigen-antibody like surfaces have been suggested for other biological systems. One of these is the relationship between enzymes and their substrates, such as the relations between specific glucosidases and chemically modified glucosides recently reviewed by Pigman ('44). From the antigen-antibody like reactions between the surface and underlying substances in certain invertebrate eggs, Tyler ('40) has suggested that such complementary structures may be an important feature in the architecture of all cells. On the basis of physical-chemical considerations, Pauling and Delbrück ('40) postulated that most biological syntheses involved the building of complementary surfaces, pointing out that complementary surfaces can be identical under certain circumstances.

While there is no direct evidence indicating that genic specificity resides in the unique surface configuration of the gene, there are several reasons for making that inference. In the first place, the specific active surfaces of enzymes (Tatum and Beadle, '45) and naturally occurring antigens (references in Emerson, '44) are themselves gene controlled. The simplest interpretation would be that surfaces of

the enzymes and antigens are derived from the surfaces of the genes involved. In the second place, genic specificity is transmitted through molecules of different chemical make-up. For example, the nucleoproteins in the chromosomes of the fish change during the ripening of the sperm from nucleo-histones to nucleo-protamines with no break in the continuity of genic specificity. This situation seems to me to be readily understood on the basis of surface configurations, especially when we recall that polysaccharide antigens determine the surfaces of gamma-globulin antibodies.

There are two general routes by which the surface structure of a gene could be transmitted in gene reproduction, and from the gene to the enzyme, or antigen. The gene could reproduce by forming a complementary, antibody-like template upon which the surface of the new gene is synthesized, or the specific surface could be copied directly if the gene has a structure like that proposed by Delbrück ('41; cf. Gulick, '44). The primary gene product in the cytoplasm could obtain its specific surface in either of these ways.

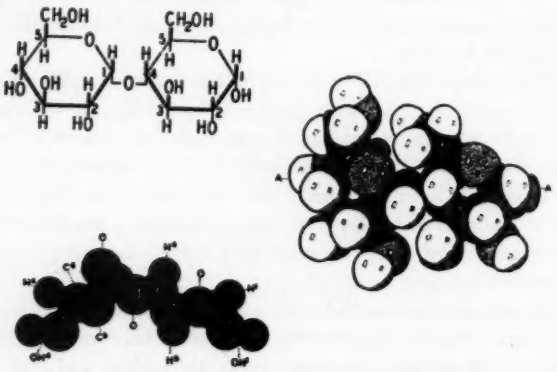

Fig. 1. Maltose molecule; upper left, structural formula; center right, surface view of model in same orientation as formula (black, carbon; stippled, oxygen; others, hydrogen); lower left, section through model, along line A—A.

These routes by which specific surfaces can be transmitted are shown schematically in figs. 1, 2, and 3, in which the gene determining the active surface of the enzyme maltase is used as an example. In the first figure a molecule of maltose is represented in three ways, by the structural formula, by a surface view of a model, and by a section through the model.

From what is known about the relationships between certain groups on the maltose molecule and the specific enzymes, it is possible to guess the surface of the sugar molecule which may be associated with the enzyme surface. Of special importance are the alpha position of the glucoside linkage (differentiating maltose from cellobiose) and the position of the hydroxyl group on carbon atom 4 of the

glucoside residue (which distinguishes glucosides from galactosides). Judging by the specificities of beta-glucosides (Pigman, '44), relatively minor substitutions on carbon atoms 2 and 3 of the glucoside radical would destroy the specificity, whereas minor substitutions on carbon atom 6 of this residue, or fairly substantial substitutions on the other glucose residue, should only lessen the specificity. These considerations make it seem probable that the enzyme attaches either to the upper surface (as illustrated in fig. 1), or to the lower surface. The upper surface has been chosen for purposes of illustration.

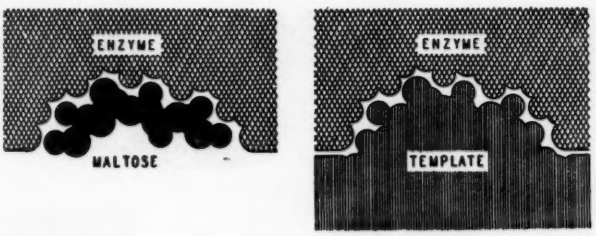

Fig. 2. Complementary surfaces: left, section through maltase (ENZYME) with associated maltose molecule; right, maltase and complementary template.

The diagram at the left in fig. 2 represents a section through a molecule of the enzyme maltase which has a molecule of maltose associated with its specific active surface. The surface drawn for the enzyme is not intended to be exact in any way, but is intended to represent a complementary relationship to the surface of maltose in the same sense as antigen and antibody molecules are complementary. At the right in this figure is a section through the same enzyme and a complementary template upon which the surface configuration of the enzyme might be determined. As drawn, the surface of the template is identical to the corresponding surface of maltose. They are not identical, one being a carbohydrate, the other presumably a nucleoprotein, but they must be similar to the extent that both are complementary to the surface of the enzyme.

Figure 3 is a scheme illustrating the possible routes of gene reproduction and gene action. The specific surface configuration of the gene (G) may be reproduced directly as shown by the dotted line, or indirectly through the intervention of a complementary template (T^G). If both gene and template are part of the genic material it is purely a matter of convenience which is called gene and which template. If the enzyme (E) has a surface configuration identical to that of the gene (upper half of figure) it may obtain this surface through a complementary template (T^E) or directly (dotted line), depending upon the structure and mode of synthesis of the molecules involved. The lower part of the figure shows how the enzyme might have a surface complementary to that of the gene, in which case there could be no surface in the genic material identical to that of the enzyme

unless the gene reproduces by means of a template.

Except for intermediate steps in gene action (which must duplicate the steps illustrated), this diagram exhausts the possible ways by which specific surfaces can be transmitted according to the postulates outlined above. It should be possible to distinguish between these alternative routes experimentally. Sturtevant ('44) and I (Emerson, '44) have pointed out how antibodies to natural antigens could cause mutations, provided the gene and antigen have similar surfaces, and we have interpreted certain examples on that basis. The argument used is briefly this:

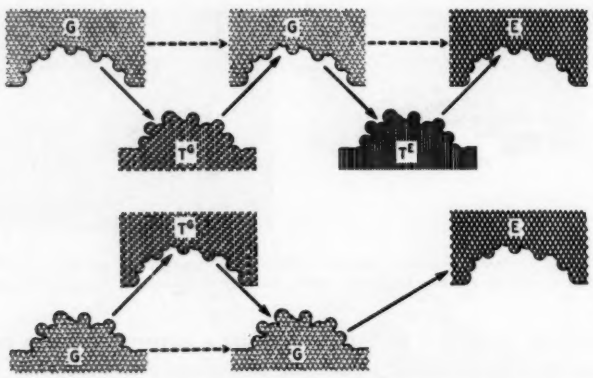

Fig. 3. Possible routes by which specific surfaces can be transmitted from gene (G) to gene and from gene to enzyme (E). T^G is a complementary gene template, T^E, an enzyme template.

that since the specificity of an antigen is genetically determined, gene and antigen may have identical surfaces, in which case antibodies developed against that antigen could combine with the corresponding face of the gene. The presence of the antibody molecule on the surface of the gene would so change that surface that the gene could no longer make an exact copy of itself, but would either fail to reproduce, or would produce a new gene with an altered surface. Either of these results would be recognizable as a mutation since the descendants of this cell could no longer elaborate the antigen in question. Experimental evidence is still inadequate on this point as there is no one case in which antibodies to a particular antigen have been shown to induce mutations in the gene responsible for the production of that antigen. If such evidence is forthcoming, it would indicate that the surface of the antigen is duplicated in either the gene itself or in the gene template.

If this scheme for the induction of mutations by antibodies should be correct, it should be possible to accomplish the same result more simply. For example, it is known that maltase activity can be inhibited by the presence of an excess of glucose. The glucose molecule fits into part of the active enzyme surface, and

when present in excess there is usually a molecule of glucose in the way of the maltose molecules which generally fit into this surface. By using very high concentrations of glucose it should be possible to have one of its molecules associated with the corresponding surface of the gene at the time of gene reproduction, resulting in mutation in the same way as when antibodies are present. We have made a few attempts along these lines by treating *Neurospora* with high concentrations of different sugars, analogues known to give substrate inhibition with certain enzymes, etc. Except for one mutation to be discussed later, the method has not proved too satisfactory, resulting usually in a great deal of sterility.

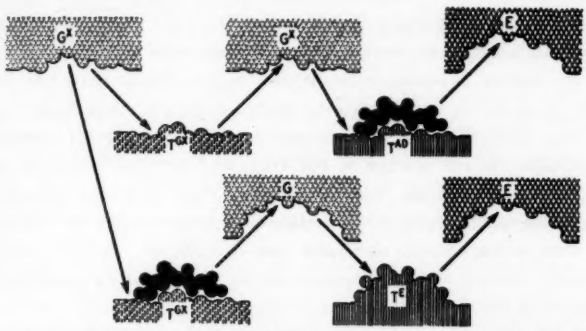

Fig. 4. Formation of adaptive enzymes: T^{AD}, adaptive template (maltose shown in in black); G^X, gene; T^{GX}, gene template; E, enzyme (maltase); G, mutated gene; T^E, enzyme (maltase) template. Further explanation in text.

The scheme just outlined can be extended to account for certain adaptive phenomena. The upper half of fig. 4 illustrates "cytoplasmic" or non-inherited adaptation. G^X represents a different gene from the one previously illustrated, and is shown as reproducing itself by means of a template (T^{GX}). The role of this gene is to produce a nucleoprotein template (T^{AD}) in the cytoplasm. In the presence of maltose, this template attaches to a molecule of maltose by its reverse side, and the surface of the template is altered in much the same way as the surface of an antigen is altered by the presence of a haptenic group. The enzyme that is synthesized on this template-plus-maltose will have a surface configuration complementary to the surface of maltose, which gives it the surface structure of the enzyme maltase (E). In this instance, maltase is an adaptive enzyme which is produced only in the presence of maltose. This is the way I had pictured the production of the killer substance in *Paramecium* as reported by Sonneborn ('43a, '43b), but from the additional evidence he has now presented (Sonneborn, '45) it is apparent that the scheme I have outlined will not account for all of his observations. Lindegren, Spiegelman and Lindegren ('44) have shown that genes are involved in producing a background for adaptive melibiose fermentation by yeast, giving a situation that fits the scheme outlined here, but to account for the addi-

tional observations just reported by Spiegelman ('45) it would be necessary to have the adaptive template (cytoplasmic factor) self-reproducing, as he has suggested.

The lower half of the same diagram (fig. 4) shows what must happen to the gene responsible for the adaptive structure under proper circumstances. If the organism is cultured in very high concentrations of maltose, enough molecules of the sugar should get into the nucleus so that some could combine with the gene-template (T^{GX}), and the gene then constructed on this template should produce maltase ever after, even in the absence of maltose.

Two examples that may represent mutations of this sort have recently turned up in our laboratory. By culturing *Neurospora* in the presence of sulfanilamide, Cushing (unpublished) was able to adapt a strain to the extent that conidia would germinate on higher concentrations of the sulfanilamide than the non-adapted strain, and, at a given concentration, mycelial growth was more rapid in the adapted strain. The adaptive characteristics were only partially maintained after a single subculture in the absence of the drug, and completely lost on outcrossing, indicating that the adaptation was not genetic. After growing the adapted strain on still higher concentrations of sulfanilamide, a heterocaryon was obtained which contained some normal nuclei and some mutated nuclei. Isolated mutant strains were extremely tolerant to sulfanilamide and in some respects made better growth in the presence of the drug than in its absence.

The second example is the one mutation obtained following treatment of *Neurospora* with a molecular solution of lactose. Lactase is an adaptive enzyme in *Neurospora* in that it is not produced in the absence of the specific substrate. The mutant differed from wild type in that it grew poorly on all carbon sources, but, in contrast to wild type, it grew just as well on lactose as on glucose, suggesting that lactase might be produced irrespective of specific substrate. Tests are now under way to determine if this is the case. The experiments to date do not distinguish between induced and spontaneous mutations.

While there is little available experimental material bearing on the mechanisms outlined, it may be useful to have them presented at this time. The scheme has many postulated steps, but each is fairly reasonable in the light of our present knowledge, and it does give a rather unified picture. There is the further point that the scheme should be amenable to experimental attack, and if the experiments are successful we have a way of learning something about genes from a different approach by making use of the methods of enzyme chemistry and immunochemistry.

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DISCUSSION

Following Reading of Lindegren's and Tatum and Beadle's Papers

Dr. Demerec: Yesterday I attended a seminar at the University of Missouri where Dr. Briggs from the University of California discussed the disease resistance of plants. He described four genes which determine resistance to smut in wheat and three out of those four are located in one chromosome. Now there are several instances where genes of like action are located in the same chromosome, and we have a number of cases where genes of very similar action are located very close together. I wonder if Dr. Beadle has any indication of instances where genes closely similar are located close together.

Dr. Beadle: There are a number of cases where they seem to be associated in ways that you might not expect. For example, there are two different albino strains that appear to be genetically different, yet they are so close together they practically never cross over. There is one chromosome, you may remember, concerned with three pyrimidineless mutants and I would hesitate to say whether they are chance distributions or not. You cannot take the chromosome map as a random sample. By our procedure we tend to pick up a non-random assortment of linkages.

Question: In some cases several genes may be involved in one very simple step of the over-all synthesis. That seems to indicate that those genes work together in the manufacture of the enzyme for that step. Is there any evidence that some other substance may be supplied which would enter into the formation of the enzyme?

Dr. Tatum: We have no evidence on that question. In the cases illustrated in which there are four mutations concerning the synthesis of arginine there must be four different steps involved.

Dr. Muller: Where you have two or more different mutations affecting the same synthesis and the substances are diffusible can you always get growth if you give the product of one of them to the other?

Dr. Beadle: Yes, if the substances are stable.

Dr. Stadler: Once you find the essential substance that is lacking how do you keep the culture growing?

Dr. Tatum: On a minimum amount; the cultures are given enough to grow, but no excess.

Dr. Cori: These intermediate products accumulate. To have them accumulate you stop formation of the final product. Why should the reaction continue in the intermediary stages if the final product is supplied?

Dr. Beadle: You don't have intermediates formed if you give a sufficient

amount of the final product, just enough to tease it a little. If you give plenty of adenine no pigment whatever is found, but if you give it just the right minimal amount, a large amount of purple pigment is formed.

DR. GODDARD: Dr. Tatum, how long does it take to bring about an adaptation that you can detect, a matter of hours, days, or longer?

DR. TATUM: Ordinarily, it is a matter of days, not hours.

DR. GODDARD: Is there any relation between the amount of mycelium and adaptation time?

DR. TATUM: I think there is; probably larger amounts of mycelium adapt more rapidly.

DR. GODDARD: I think there is a conceivable biochemical change important in adaptation and related to the volume of the tissue versus volume of the liquid. If you started from A and went to Z and had no intermediates, it would take an extraordinarily long time for anything to accumulate; time measured in terms of hours and not minutes. If, in a mutant, you suddenly have blocked one stage it might take a good many hours to build up sufficient concentration so a new reaction can start. This type of adaptation could not involve changes in existing enzymes or genes, but a real change in components. As James Franck pointed out, concerning a reaction which starts within a minute or so after illumination occurs, if all the intermediates between CO_2 and carbohydrates had to be built up it would take eight hours. As a consequence, one knows the intermediates must be present.

DR. DELBRÜCK: Can you tell us something about reverse mutations: whether they occur and can be recognized?

DR. BEADLE: The main difficulty in answering your question is the experimental difficulty in telling the difference between contamination and reverse mutation unless you have a special set-up because, of course, you would expect to get a wild type in reversion and you could also expect contamination by wild type.

DR. DELBRÜCK: It seems to me if this mutant can revert it should have a well-defined reverse mutation rate and should be distinguishable from an erratic contamination.

DR. BEADLE: If the mutation rate is high enough.

DR. DELBRÜCK: For spontaneous reverse mutation one could use Roepke's method of growing the mutants in the presence of minimal amounts of the missing factor and then see whether you finally get rapid growth.

DR. BEADLE: We have not done that extensively.

DR. HOLLAENDER: Have you found any difference between spontaneous mutation rates and rates produced by different types of radiation?

DR. BEADLE: We have not studied spontaneous mutation rate because, so far, we have not concerned ourselves with problems concerning mutation rate in

general. For one thing, we figured you could do a better job; also, we thought that the amount of testing we would have to do to determine the spontaneous mutation rate was so great we hesitated to undertake it.

DR. TATUM: I have a remark in connection with Dr. Goddard's suggestion. There is one mutant strain which requires thiazole and makes pyrimidine. This does not adapt to the slightest extent, no matter how long it is left. On the other hand, the thiaminless mutant strain which requires the intact molecule and which makes both intermediates adapts extraordinarily well. That might support this idea. However, in another instance involving pantothenic acid adaptation did not occur. So there are differences.

DR. GREENSTEIN: I would like to point out the very remarkable parallel between these effects that Dr. Tatum and Dr. Beadle have obtained and changes in neoplastic tissues. If one compares functions of normal living cells with those of tumors one finds similar reactions, but one must also assume that newer functions are induced. I wonder if Dr. Tatum and Dr. Beadle considered the possibility that mutant effects may have induced the formation of new functions, perhaps unnecessary, but present nevertheless.

DR. TATUM: I think there are pertinent examples of the formation of new substances in the production and utilization of intermediates and the production of pigments. It is perfectly possible that those are merely instances which we picked up; perhaps a whole series of new reactions are instigated by means of blocked reactions.

DR. MULLER: Would it be possible to test blocking any of the enzymes by anti-bodies?

DR. TATUM: I think that is quite possible to do. Perhaps Dr. Emerson has some comments.

DR. EMERSON: It would be easier to block with substrate; antibodies usually are not directed against the active groups on the enzymes.

DR. EDGAR ANDERSON: I would like to ask Dr. Beadle if he has information on chiasma frequencies.

DR. BEADLE: No, I haven't any, and I think Dr. McClintock has not made actual counts, although I think she could give you an estimate. I suppose the average is perhaps between one and two, possibly around two, but that is just a very rough guess.

DR. STADLER: I notice in the general discussion by Dr. Tatum that when there are, say, four genes affecting a step or sequence of steps, it is apparently assumed that it must involve four or more enzymes. I wonder if the results contradict the assumption that production of a single enzyme could be affected by two, three, or four genes?

DR. TATUM: We feel quite certain that there are a number of instances in which a number of genes will affect a common enzyme.

* * *

FOLLOWING READING OF SPIEGELMAN'S PAPER

DR. GODDARD: It would be of some importance to get at the nature of the enzyme-substrate compound. Do you know the Michaelis constant? Is the amount of galactose tied up with galactozymase dependent upon the galactose concentration?

DR. SPIEGELMAN: In attempting to obtain rate-concentration curves we ran into several difficulties. At high concentrations there is an inhibition of adaptation, probably osmotic in nature. At low concentrations it is difficult to keep the concentrations constant due to the preadaptive aerobic utilization of the galactose.

DR. GODDARD: The low concentration experiments are critical. Perhaps one might place the culture in a collodion bag suspended in a large volume of the galactose solution.

DR. BEADLE: Considering the four positive spores which segregate from the first hybrid, have you been able to demonstrate any difference at all in the two types, or have you looked for such a difference?

DR. SPIEGELMAN: As a matter of fact, a great deal of effort was expended in trying to find such differences. None was detectable. I might mention in passing that we tried to see whether the rates of adaptations differed between a haploid carrying two mel+ genes and one carrying only one such gene. Here again no difference could be established.

DR. BEADLE: In this diagram you have presented of gene action is it possible to suppose that the gene is originally determining the specificity of "P" which in turn somehow acts as a pattern for "E"? An analogous situation might be the transformation of pepsinogen into pepsin.

DR. SPIEGELMAN: I see no objection against a supposition of this kind. It will be noted, however, that it grants even more than I ask, that even in the presence of the controlling gene there exists a step in the chain which is autocatalytic and completely free of primary gene action.

DR. MULLER: I should like to raise the question of the specificity of the synthesis of "E", especially with relation to the gene. How likely is it to depend on factors other than this gene and "P"?

DR. SPIEGELMAN: You mean besides the mel+ gene?

DR. MULLER: Yes, other than the gene whose absence deprives it of the adaptation possibility.

DR. STADLER: I think the point mentioned by Dr. Muller is the one with which we are all concerned. It seems to me that the crucial question which must be considered here is whether we must, as a consequence of these experiments, add to our array of self-reproducing units a fundamentally new one; the self-reproducing enzyme molecule. Or, can we by not too complex or artificial a system construct a picture according to which we might account for the experiments without postulating a new self-reproducing unit? I know that Dr. Spiegel-

man has considered this question and I should like to ask him what assumptions he thinks are necessary to make it possible to explain the experimental results on a genic basis.

DR. SPIEGELMAN: We may assume the existence of a gene "X", different from either of the two mel+ genes already mentioned and capable, under certain conditions, of performing the same function. We must, in addition, ascribe the following properties to the "X" gene: it can only perform its function if kept in continuous contact with the enzyme. Thus the "X" gene would require the presence of the mel+ gene in order to start functioning. Once they were separated the "X" gene would remain functional only so long as sufficient enzymes were present in the cytoplasm; i. e., so long as the substrate was available. Another mechanism one might devise, although slightly more complicated, is that the gene "X" is stabilized directly by the substrate rather than by the enzyme. These are, I feel sure, not the simplest desirable mechanisms involving primary gene control. I am reasonably certain, however, that no mechanisms depending on genes will be able to avoid assuming that the functional stability of the controlling gene is dependent on either the substrate or the enzyme.

DR. GREENSTEIN: This hypothesis would seem to involve a serious revision of previously conceived notions of the nature of the gene. I wonder whether your reaction could not be modified somewhat by saying "P" plus "S" is equal to "E". In that way the character of your enzyme would be a function of your substrate.

DR. SPIEGELMAN: I don't see offhand that this is essentially different from the scheme I presented. The enzyme content is a function of substrate from the point of view of its stabilizing effect, and it is this aspect that I wished to emphasize. Evidence for a more direct involvement such as would be implied by the suggested modification is not available.

DR. GREENSTEIN: Are you certain that glucozymase is more stable than the galactozymase system?

DR. SPIEGELMAN: Yes. We have direct evidence in the form of both *in vitro* and *in vivo* comparisons.

DR. SONNEBORN: May I suggest that this other gene "X" we have been discussing seems to be excluded by the experiments in which removal of melibiose leads to loss of adaptability?

DR. SPIEGELMAN: The assumption of irreversible loss of function on removal of substrate (and enzyme) would explain the data.

DR. STADLER: It seems obvious that any other gene one would postulate would have to have some very artificial characters. The very nature of these characteristics could, by their artificiality, carry us a long way toward the necessity of assuming self-reproducing enzyme molecules. We may perhaps simplify the question under discussion if we stress the comparative plausibility of any proposed genic mechanism with that of the self-reproducing hypothesis.

DR. SONNEBORN: There is one difficulty with the genic mechanism. The "X" gene can apparently react to substrate when it is in the presence of the

mel+ gene but it loses that capacity when both the mel+ gene and the substrate are removed.

DR. SPIEGELMAN: If one is bent on retaining the gene mechanism it is not too difficult to get around this dilemma. One could assume that there is a sort of "position effect" in terms of a diffusible substance made by the mel+ gene required by the gene "X" for functional activity in the absence of substrate. Even simpler, it seems to me, would be to explain it in terms of interaction between gene "X" and enzyme, which latter we know is mediated by the mel+ gene.

DR. DELBRÜCK: Omitting for the moment the question of how one can avoid the self-duplication of enzymes, I should like to discuss another aspect of the problem. One property you have assumed which perhaps might at first glance appear peculiar is that the transformation from "P" to "E" can be catalyzed by two different agents, gene and enzyme. The first thing one would suspect is that perhaps "G" and "E" are very similar, a suspicion you have probably entertained. I might add that aside from these experiments and your formulation it is a suspicion one might have in any case, in view of the close correlation between gene and enzyme presented this morning by Dr. Beadle and Dr. Tatum. We would picture the present instance then in the following terms: you have a gene "G" which is stable so long as it remains in the nucleus. It produces an enzyme "E", similar to itself. When the enzyme gets into the cytoplasm it becomes unstable, a condition which, as you pointed out in the beginning of your talk, may be a very general one. Now if this unstable enzyme is stabilized by substrate then it can, in the cytoplasm, replace the gene. This seems to me a very fruitful picture. I do not, however, wish to lead you away from the search for alternative explanations.

DR. SPIEGELMAN: I should like to point out one difficulty I have encountered in trying to push the analysis along the lines suggested by Dr. Delbrück. If the genes and the enzymes they produce are similar why is it that the former are stable, whereas the latter are not? In terms of modern biochemical concepts the instability of the enzyme in the cytoplasm is the easier of the two to understand. All the components in the cytoplasm are in a state of flux and any given one can maintain itself only by balancing the rate of its disappearance with an equal rate of resynthesis. Here the substrate performs two functions, stabilizing the enzyme molecules and providing energy for synthetic activity. We have more or less direct evidence for this view. The rate of disappearance of enzyme in the absence of its substrate is proportional to the over-all metabolic turnover of the cells. Such unstable enzymes can be stabilized by depressing metabolic activity with, e. g., anaerobiosis or with sodium azide which prevents nitrogen assimilation. One way of explaining the stability of the gene on the same basis would be to assume that it is outside the metabolic cycle and that it alone of all protoplasmic units does not undergo continual breakdown and resynthesis. This raises the obvious difficulty of being forced, in a sense, to remove genes from the effects of

reactions which they control and with which they must necessarily be in intimate physiological contact.

DR. CORI: One fundamental question here is what is meant by reproduction of enzymes. What ideas do you have on this with respect to the mechanism and the nature of the precursor?

DR. SPIEGELMAN: I cannot go further at present than to say that the enzyme influences its own production. The details of the mechanism must await the elucidation of the nature of the precursor. With respect to the latter it seems to me unlikely that we are dealing here with synthesis all the way from the amino acids. It appears more likely that the precursor is an indifferent protein.

DR. BEADLE: I don't quite see the reason for the anxiety to avoid the concept of self-duplication of enzymes. In this connection, I should like to bring up again the pepsinogen-pepsin transformation and its relation to this problem.

DR. MULLER: I should differ with Dr. Beadle in just one respect. I think there is evidence here of self-reproduction; something akin to the gene. However, we do not know how general this phenomenon is. The concept is sufficiently novel to require more proof before its generalization is accepted. With respect to the pepsin formation it must be noted that it requires a very specific precursor, pepsinogen.

DR. BEADLE: How do we know that we do not have a specific precursor here much like pepsinogen? Wouldn't we then have what appears to be self-duplication in terms of transformation from precursor?

DR. GREENSTEIN: Pepsinogen is an inactive form which can be activated by either pepsin or hydrochloric acid.

DR. BEADLE: Couldn't "P" be an inactive form of "E"?

DR. GREENSTEIN: Perhaps, but it is an inactive form whose ultimate activity is dependent upon the types of substrates available at any one time. If we regard the proteins as the precursors, then their final enzymatic activity would depend on the available substrates and the genes would provide a *modus operandi* to allow it to proceed in the direction permitted by the existent conditions.

DR. BEADLE: Perhaps I should put the question in a different way. Is it possible that in the self-duplication of genes in general there exists a common inactive form which can be transformed in many ways to make different genes?

DR. MULLER: All the evidence we have points to a very general "precursor" for genes. The most essential property of a gene is expressed in its capacity to produce an exact duplicate irrespective, within wide limits, of associated genes or environmental conditions.

DR. SPIEGELMAN: There are several aspects of the pepsinogen transformation as an analogy that I would like to mention. The formation of pepsin from pepsinogen is spontaneous if pepsin and hydrogen ions are available, i. e., no energy is required. This is not true for the enzymes we are considering. Removal of the source of energy supply leads to cessation of enzyme formation. As far as the genic aspects of the problem are concerned, it seems to me little is gained by

the analogy. Pepsinogen already contains inherently all the specificity of pepsin. Thus the analogy would say that what we are observing in the kinetics of adaptation is a simple autocatalytic activation of inactive galactozymase or melibiozymase. Presumably the main function of the gene is specificity determination of the enzyme it controls. We would thus leave unexplained how the inactive forms of the enzymes with their inherent specificities could be synthesized in the absence of their specific genes.

DR. LINDEGREN: Isn't it true that a direct application of this analogy would mean that there was always available a considerable amount of this specific precursor even in the unadapted cell? This does not seem to be a reasonable situation.

DR. SPIEGELMAN: In this connection we might mention that when one tries to induce two enzymes simultaneously a competitive interaction can be established. This could be interpreted as competition for a common substrate which goes into the formation of the two different enzymes and would argue against any great specificity of the precursor.

DR. TATUM: It could be a competition for a limited source of energy.

DR. SPIEGELMAN: That is certainly true.

DR. HERSHEY: And it might be noted that shifting the gene action to the reaction forming "P" does not change the nature of the question.

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FOLLOWING READING OF HOLLAENDER'S PAPER

DR. RAPER: I might amplify just a bit what Dr. Hollaender was saying about this Aspergillus work. To test organisms for itaconic acid is time-consuming. Dr. Beadle does not know how fortunate he is in tracing down certain vitamins. Among the large number of cultures tested were about fourteen we thought particularly interesting. One culture did not increase the yield of total acid, but gave itaconic acid of exceptionally high purity. The other thirteen cultures did give total yields higher than the average for all of the controls which were run along with them, and of this number there were one or two that gave increases of 15 to 20 per cent. That is not a spectacular increase, but it is significant.

At the same time we have been isolating strains from nature. About 330 or so of these have been tested, and we now have a dozen or more that are better than our original stock. This merely goes to show that we did not have the best organisms to start with.

Dr. Hollaender alluded to the fact that they came from Texas. The majority have come from soils obtained from Texas, Arizona, New Mexico, and whether it means anything or not, Calcutta, India, and Western Australia. The temperatures are fairly high and the climate rather dry, a fact that may be of interest to you.

I would like to ask Dr. Hollaender whether the penicillin production tests

were run in submerged culture.

DR. HOLLAENDER: Yes.

DR. RAPER: Assays showed maximum yields at three days?

DR. HOLLAENDER: Yes.

DR. RAPER: Maximum yields at three days are a little unusual in the type of equipment with which we work. I do not know whether it is Dr. Beadle's experience or not.

DR. BEADLE: We would agree with you that they would come later.

DR. RAPER: I would raise one slight question about some of the yields. On the second-, third-, and fourth-day assays you would not expect the tremendous jump and then the abrupt fall shown in some of these figures. They jump from 18 to 60 to 20 on the second, third and fourth days respectively. One would expect that the slopes of that curve would be more gradual as a general thing.

DR. HOLLAENDER: We have data on five-day cultures that show fairly high yields but not so high as the three-day cultures.

DR. MULLER: If we assume that a given product, such as penicillin, has some adaptive value to the organism, and if we could find out the conditions under which it would help the organism, then by increasing the intensity of those conditions it might be possible to establish some sort of automatic selection. For example, if the penicillin helps to protect the Penicillium against competing bacteria, the addition of bacteria to a lot of cultures might produce such a condition.

DR. GODDARD: That was proposed some time ago and has been tried out in our laboratory and other laboratories, but we do not know of an actual stimulation or increase in productivity.

FOLLOWING READING OF GREENSTEIN'S PAPER

DR. STEINBACH: It is a rather interesting point that, as you say, you get the interaction between the nucleic acid and the globular proteins. The general tendency is to think of the protein chromosome matrix as more or less fibrous.

DR. GREENSTEIN: Since fibrous protein may at the same time be rather rolled the difference between fibrous and globular proteins may not be critical.

DR. STEINBACH: Is this interaction of certain nature? For example, if you are building up a chromosome is the nucleic acid a part of a definite pattern, stuck on the sides, or what would it be?

DR. GREENSTEIN: It is fixed only at fixed moments. The chromosome is not a stationary object; one must assume that it is changing continuously and one could expect that the physical properties of each of the components would be changed at each moment.

DR. SPIEGELMAN: What groups fix? Where does the interaction take place between the protein and the nucleic acid?

DR. GREENSTEIN: It must take place between charged groups, and this can have a marked effect upon the shape of nucleic acid.

DR. STURTEVANT: How combinations occur is of interest to geneticists who study specificity at different levels. Now, frequently it has been customary to think of genetic specificity as due largely to the protein. I take it that the current tendency is perhaps to ascribe it to the nucleic acid component. I wonder if you would be willing to throw some light on that question.

DR. GREENSTEIN: Nucleic acid, although of relatively simple structure, incapable of such alterations as proteins are, nevertheless can attach itself to certain types of proteins and produce an over-all difference in specificity. I don't quite see how a nucleic acid by itself can act; it must produce a combined effect. I think we are both coming to the conclusion that nucleic acid perhaps exerts its effect by contributing to reacting systems.

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FOLLOWING READING OF SONNEBORN'S PAPER

DR. SPIEGELMAN: I should like to suggest, Dr. Sonneborn, that the possibility of self-duplication of kappa is not entirely ruled out. Your conclusion depends on the fact that without the K gene the kappa disappears, but if we should assume that the precursor is always limiting, the amount of kappa that could be formed at any given time by self-duplication would be small. The velocity going from precursor to kappa would then be gene-controlled and you would always need the gene there to maintain kappa.

DR. SONNEBORN: Would you suggest any method by which it could be detected?

DR. SPIEGELMAN: Well, unless you know the precursor you cannot test it; that is true.

FOLLOWING READING OF GOWEN'S PAPER

DR. DELBRÜCK: You cited one instance of a spontaneous mutation, rate 1 in 180, and you determined the presence of the mutation by looking at the colony?

DR. GOWEN: Taking one of these micro-colonies and then plating out 100,000 cell groups on the assumption that you would have equal rate between the mutant type and the original type.

DR. DELBRÜCK: But you determined the character, smooth or rough, after your bacteria had grown into a colony?

DR. GOWEN: That is right.

DR. DELBRÜCK: How do you know the mutation does not occur in the colony?

DR. GOWEN: I don't. I expect it does occur in the colony.

DR. DELBRÜCK: Perhaps I misunderstood the procedure: Take one bacterium, let it grow into very small colonies; then pick out and plate individuals from the small colonies, letting each individual grow into a colony, and now you determine

whether these latter colonies are rough or smooth?

DR. GOWEN: Yes.

DR. DELBRÜCK: If the mutation rate is anywhere near 1 in 180 then all the colonies must be sectorial colonies because in each colony you grow individuals up to many millions.

DR. GOWEN: Rough and mixed, and some smooth, we think.

DR. DELBRÜCK: A colony consisting of one million smooth bacteria if your mutation rate is 1 in 180?

DR. GOWEN: I said we think they are smooth. If your mutation was toward the end of the process of course you would not be able to distinguish it.

QUESTION: How many cell generations do you have?

DR. GOWEN: About thirty minutes is the generation time.

DR. SPIEGELMAN: The experience I had some time ago with S. *aertrycke* bears out Dr. Delbrück's contention: we had a rapidly varying strain where rough gives off rough and smooth; when the mutation to the rough was high we got all rough.

DR. GOWEN: You were sure you started with rough colonies?

DR. SPIEGELMAN: Yes.

DR. GOWEN: We have not found many of those. In fact, offhand, I do not think I know of any with that particular set of characters.

DR. MULLER: It seems to me that the method will work if you have enough colonies, if you allow for the number of generations, and assume symmetrical reproduction. Can't you work it that way if you do enough colonies?

DR. GOWEN: That is right. Don't think for a minute that I think this technique is as good as micro-dissection technique, but after you work with the microscope for a year you also turn to other check methods as well.

DR. DELBRÜCK: I don't see how the micro-dissection technique can be of any help in determining mutants only recognizable in the character of the colony.

DR. GOWEN: If you grow the colony large enough, of course, you will get rough and smooth out of it and, a point I did not bring out, this change occurs in one cell generation.

DR. STURTEVANT: Perhaps I missed something. I would like to know how you keep this mutant stock in the first place.

DR. GOWEN: It is only through very rapid transfer that you may keep it. If you leave it any length of time it becomes rough very promptly. It is possible to classify the original micropipette-selected bacterium as either of the smooth type or the rough type in genetic constitution, because the mutant form leading to rough colonies is stable. All the bacteria coming from the single micropipette-isolated bacterium which is itself rough will themselves give only rough colonies. On the other hand, the bacterium forming a smooth colony is unstable. Samples taken from a colony originating from such a smooth-type bacterium will show colonies which are smooth, smooth and rough sectored, and rough.

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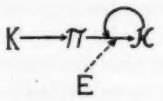
FINAL DISCUSSION

DR. MULLER: Dr. Lindegren has asked me to give a more detailed picture of the hypothesis that I suggested yesterday, when I attempted to bring his and Dr. Sonneborn's findings under the same scheme. For this purpose let us consider the diagram here shown:



Here, at the beginning, you have Dr. Sonneborn's "Killer gene", a large "K", and this produces a certain gene product, π, which in turn is required for the production of the cytoplasmic substance kappa, κ. The latter, in our present scheme, is to be considered as corresponding with the "cytogene" of Lindegren. For reasons that will appear, it seems likely that the product π is closely related in its composition to the gene K that produced it, and also to the substance kappa, or "cytogene", that follows it. If so, π may be regarded as the precursor of kappa, and would represent the intermediate step in at least two successive modifications of K. It is not absolutely necessary for the scheme, however, to assume these relationships in composition of the three substances concerned.

Now, the presence of π would not by itself be sufficient, in either Lindegren's or Sonneborn's cases, for the production of kappa. But if some kappa is already present to begin with, the reaction $\pi \rightarrow \kappa$ is activated by this kappa, as indicated in the diagram by the curved arrow that arcs upwards and backwards from kappa and impinges on the arrow leading from π to κ. Thus more kappa, or cytogene, becomes formed. If π and κ are related in composition, this reaction might be compared (as has been done by others independently) to the effect of pepsin in transforming pepsinogen into more pepsin, although it is not yet known whether this type of self-activation is common for enzymes. However, on the assumption that K, π and κ are all related, this self-converting effect of kappa could be regarded as due to kappa having preserved in itself something of the nature of a gene. It would not have a gene's more generalized ability of converting non-specific materials of the medium into material like itself, but it would have a more limited ability of thus converting a specific precursor, one which had already gone a large part of the way in the shaping of the final material.

Now, if the gene K should be removed, the chain of reactions is broken, and no more kappa can be produced. This breakage has occurred in Sonneborn's material, by mutation of the gene K to k, but nothing analogous to this mutation has yet been found in Lindegren's material. Removal of kappa also, in Sonneborn's material, breaks the reaction chain, so as to stop further kappa production. But this is not always true of what Lindegren has called the cytogene in his material. In his case, even though this cytogene is in some lines necessary for the production

of more material of its own kind, in other lines, differing from the former in a single pair of genes, which we may here designate as E versus e, the final substance or cytogene can be produced even when there is none of it present to begin with. The gene E of yeast then, or a product of it, must here be able, like kappa itself, to activate the reaction $\pi \rightarrow \kappa$ so that this reaction will go on even in the absence of initial κ. If kappa (here the cytogene) were closely related to π in composition, it would not be surprising that the conversion could be effected by other means than by kappa itself, as is true also of the pepsinogen-pepsin conversion. (The alternative scheme, not relating the two sets of results, would tend to have the cytogene similar to E instead. In the *Paramecium* material, it is as though none of the lines discovered contain E, but only e (or the absence of any comparable gene).

Another difference in the two materials lies in the fact that, for persistence of the cytogene, but not, so far as known, for that of the kappa of *Paramecia*, a certain substrate, melibiose, is required. If the cytogene can be identified with the enzyme melibiose-zymase which enables us to recognize its presence, this requirement is quite understandable, as many enzymes deteriorate rapidly in the absence of their specific substrates. But this very requirement helps us to understand also the presence of E in the yeast material, since without E the cytogene would be permanently lost every time a line of cells happened to grow in a medium lacking this specific substrate. In *Paramecia*, where there seems to be no such specific external substances which are likely to be missing from the environment, there is no need for such a gene as E to have become incorporated into the reaction system.

Of course the scheme here outlined is merely one conceivable possibility for relating the results on the two different organisms. The test of it would lie in the proof or disproof of the conception that the cytogene, like kappa, and unlike the genes of the chromosomes, continuously depends for its production on certain specific material (π), derived from or due to a particular gene.